Studies on the growth, survival, interaction and detection of potentially pathogenic *Listeria* and *Bacillus* species in infant milk formulae

by

Neil Joseph Breffni Rowan

A Thesis submitted to the University of Strathclyde in accordance with the regulations for the degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF SCIENCE

Department of Bioscience and Biotechnology
University of Strathclyde
Glasgow - Scotland

January, 1996

I dedicate this work to my
wife, Michelle and
parents, Breffni and Ruth.

I would like to thank Dr John Anderson and Dr Annette Anderton for their counselling, guidance and support throughout this study.

To the academic and technical staff in the Department of Bioscience and Biotechnology at University of Strathclyde, for their assistance and friendship.

To the Ministry of Agriculture Fisheries and Food (MAFF) and the Scottish Homes and Health Department for funding this work.

To my brothers Fergus and John and my twin sister Sharon for their love and support. I also extend my thanks to Billy and Maura Golden for their encouragement and to Mr. Paddy Golden for his friendship and hospitality during my writing up period in Ireland.

To my aunt Clare and uncle Paul, for their friendship, kindness, companionship and direction during my earlier years of study at University College Galway.

TABLE OF CONTENTS

Abstract

The heat resistance of *L. monocytogenes* may be significantly improved by modification of the cell's cultivation and heat treatment environments. The recovery of heat treated *Listeria* cells was influenced by the cultivating, heating, diluting, enriching and enumerating conditions.

Rough cell and colony forms of *L. monocytogenes* emerged during these thermal studies, which were successfully isolated and identified using current enrichment/enumeration media and diagnostic kits. Both morphological forms exhibited thermal death rate curves, although the rough form was revealed to be more heat resistant. Determination of the cell's thermotolerance was achieved by linearisation of the survivor curves via the formula $(\log No - \log N)^a = kt + c$. Serotype 4b strains of *L. monocytogenes* were shown to be more thermotolerant and the efficacy of the recommended FDA and USDA enrichment methods was improved by the use of a primary non-selective enrichment stage.

A practical method for the identification of *Bacillus* spp. was developed. A detailed microbiological analysis of 125 infant milk formulae was performed which identified various preparation and storage conditions that influenced the type and number of organisms present. Some *Bacillus cereus II* isolates produced diarrhoeal enterotoxin in reconstituted infant formulae (supplemented with maltodextrin) at ambient and near refrigeration temperatures. *Bacillus* spp. were recovered from these products which were previously implicated in either clinical infections and/or food related illnesses, while *L. monocytogenes* was not detected. The efficacy of current cleaning and sterilisation procedures to eliminate *B. cereus* from contaminated infant feeding bottles was evaluated.

Antagonistic studies between 178 *Bacillus* spp. (isolated from reconstituted infant formulae) and *L. monocytogenes* revealed that 22.5% exhibited various levels of antagonism against both morphological forms of *Listeria*. This antagonistic activity was demonstrated in solid, semi-solid and liquid media. These antagonistic *Bacillus* exhibited various levels of antagonism against other members of the genus *Listeria*. The study was concluded with an investigation into the nature of this antagonism.

1 Introduction.

Listeria monocytogenes, a facultative intracellular pathogen, has been implicated as the causative agent in numerous cases of human and animal illness going back as early as 1926. In recent years, epidemiological evidence has conclusively shown that this non-spore forming Gram positive rod has been the aetiological agent in a large number of food related illnesses. Indeed, it has become apparent that the main route of transmission is via contaminated foods, and whether judged by economic or public health impact, this bacterium has become one of the most important foodborne organisms of the last two decades.

Due to the ubiquitous distribution of *L. monocytogenes* in the environment, there has been a wide variety of food groups implicated in foodborne outbreaks of listeriosis, these include, raw and pasteurised milk, soft cheese, poultry, coleslaw, paté, seafood and jellied pork tongue. A cause for particular concern is the uncertainty surrounding the organisms heat resistance capability, the actual number of cells required to initiate an infection, its ability to proliferate at refrigeration temperatures, and the consumer groups most susceptible to its infection (i.e. pregnant women, neonates and perinates, immunocompromised patients, and the elderly). Furthermore, the mortality rate among members of these high risk groups contracting listeriosis has been shown to be high.

While the main thrust of scientific research has been focused on the suitability of certain food groups, such as dairy, vegetables and/or meat products, to support the growth and development of *L. monocytogenes*, little or no work has been carried out on establishing whether this organism can be successfully recovered from reconstituted infant milk formulae (IMF), which would have obvious consumer health implications.

Breast milk provides an immunological and nutritional food which is considered optimal for the subsequent development of the infant; however, circumstances (clinical or otherwise) may arise whereby the parent may no longer be able or wish to breast feed and under such conditions a suitable alternative food in the form of IMF must be used. Owing to the susceptibility of infants to enteric pathogens (which may

be harboured in contaminated IMF products), the objective of this research was to establish the type, number and concentration of microorganisms present in IMF products under various conditions of reconstitution, cooling and storage, in addition to the identification and implementation of optimal enrichment conditions previously shown to enhance the recovery of healthy and heat injured *Listeria* cells.

It is well established that the process temperatures employed in the manufacture of spray dried IMF are considered bacteriocidal to non-spore forming bacteria. Therefore, the first section of this project was devoted to elucidating and implementing a controlled set of conditions which optimised the heat resistance and subsequent recovery of heat treated *L. monocytogenes* cells from IMF products. In the course of these thermal studies, irregular colony morphological variants of *L. monocytogenes* occurred (which were designated as rough). Furthermore, it was demonstrated that both surface culture forms exhibited a non exponential thermal death rate on heating and that the rough form was more heat resistant compared to that of the standard smooth form when heat treated under similar conditions. Linearisation of the curved thermal death rate survivor curves was achieved through the application of a linearisation formula and subsequent thermal death rate kinetic data was calculated. The efficacy of the standard recommended techniques (with and without modifications) to detect both heat treated pleomorphic forms of *L. monocytogenes* were challenged and evaluated. The information gained through the enrichment of sublethally injured and uninjured *Listeria* cells was subsequently used to analyse IMF products for the presence of this psychrotrophic organism.

When this project was initiated, *L. monocytogenes* had been established as a major foodborne pathogen. As a direct result of this heightened attention, a large number of *Listeria* related products (e.g. identification kits, enrichment media etc.) became commercially available. During the course of these thermal studies (and/or subsequent investigations) the efficacy and reliability of these product were challenged with both heat stressed morphological culture forms of *L. monocytogenes*. This section concluded with a controlled sequence of experiments illustrating the development of rough colonies compared to the smooth surface culture form via the image analysis technique.

The next section of the project involved a detailed bacteriological analysis of 100 commercially available IMF products, with an aim to establishing the type, number and concentration (CFU ml^{-1}) of indigenous microorganisms present (in addition to the possible presence of *L. monocytogenes*). These infant milk powders were reconstituted under various conditions of reconstitution, cooling and storage temperatures over different storage periods, in order to establish the change in microbial profiles and to observe any subsequent microbial interactions. The IMF powders were also reconstituted at the recommended temperature of 56°C, which was the same temperature employed to investigate the heat resistance capabilities of the pleomorphic colony forms of *L. monocytogenes*. This detailed investigation was carried out as very little information was available in the literature regarding the bacteriological quality and/or methods of reconstitution and storage of rehydrated infant milk powders.

The indigenous microbiological flora of dried milk formulae consist of endospore-forming bacteria of the genus *Bacillus*. As the identification of *Bacillus* to the species level is frought with difficulty (i.e. there are 12 other bacterial genera which form endospores in addition to over 1300 mesophilic members of the genus *Bacillus*), a detailed study was initially carried out to establish a suitable identification method which would confidently confirm the identity of presumptive spore-formers to a *Bacillus* species level.

During the latter stages of this investigation, 25 reconstituted IMF samples collected from a hospital in Glasgow (Scotland) were analysed for microbial quality under different storage conditions.

Evidence in the literature suggests that certain members of the *Bacillus* genus may be capable of growth at psychrotrophic temperatures which would obviously influence the microbial quality of refrigerated products. Furthermore, certain strains of *Bacillus cereus* type II are capable of producing an enterotoxin which has been implicated in a number of foodborne illnesses where the patients suffer from bouts of severe diarrhoea. Thus, a bacterial culture collection was constructed which consisted of 178 *Bacillus* species isolated from IMF products, of which two *B. cereus II* isolates (1.1%) were identified as potential threats to consumer safety, being capable of

growth over the temperature range 8 to 35°C in addition to the production of a diarrhoeal enterotoxin.

In relation to the maintenance of infant health and safety, the project also highlighted the potential threat of supplementing IMF products with hydrolysed starch derivatives (e.g. maltodextrin). This research revealed that infant milk products which do not contain the supplemented maltodextrin do not support subsequent production of the diarrhoeal enterotoxin by indigenous *B. cereus* II isolates, whereas the IMF containing maltodextrin demonstrated high levels of this diarrhoegenic enterotoxin. This section of the project concluded with an evaluation of the efficacy of current infant feeding bottle cleaning and commercial sterilising methods to reduce the microbial load of inadequately stored bottles containing reconstituted IMF.

In the event that *L. monocytogenes* could either survive the harsh temperatures employed during the IMF spray drying process and/or infiltrate these powdered products as a result of post process contamination, an investigation was carried out to establish whether the indigenous IMF microbiological flora would co-habit the same environment without demonstrating any antagonistic interaction. Indeed, there is considerable interest in microorganisms that interact antagonistically with *L. monocytogenes* and only one other report has appeared in the literature where a *Bacillus spp.* inhibited the growth of this potential foodborne pathogen.

Consequently, the remaining section focused on the ability of each member of the newly constructed IMF culture collection, to interact antagonistically with both colony forms of *L. monocytogenes*. Indeed, the research identified 40 *Bacillus* isolates (2.5% of the IMF culture collection) capable of exhibiting an antagonistic activity towards the two morphological forms of *L. monocytogenes* and against other members of the genus *Listeria* via direct and deferred antagonistic methods.

The project was concluded with a detailed investigation as to the nature of the antagonistic interaction between the *Bacillus* and *Listeria* species in both reconstituted infant milk formula and laboratory based culture media.

In summary, the practical work in this thesis covers the following 15 areas:

1. Cultural studies of standard and pleomorphic surface cultures of *Listeria monocytogenes*.
2. Identification and implementation of conditions shown to enhance the heat resistance of *L. moncytogenes* in infant milk formula (IMF).
3. Studies on the emergence of colony variants of *L. monocytogenes* as a direct result of heating.
4. Studies on the emergence of smooth and rough culture forms of *L. monocytogenes* via the image analysis technique.
5. Linearisation of non-logarithmic thermal death rate curves and a comparison of the thermotolerance capability between different strains and/or pleomorphic forms of *L. monocytogenes.*
6. Evaluation of the standard enrichment techniques for the recovery of heat treated cells from both culture forms of *L. monocytogenes*.
7. Investigation to establish and validate a practical method for the identification of *Bacillus* species in IMF products.
8. Studies on the effects of reconstitution, cooling and storage conditions on the type, number and concentration of indigenous microbiological flora present in 100 IMF products.
9. Studies on the microbiological quality of reconstituted IMF prepared at a hospital in Glasgow (Scotland).
10. Studies on the ability of *B. cereus II* isolated from IMF to produce diarrhoeal enterotoxin at either ambient and/or near refrigeration (5°C) temperatures in laboratory based media and reconstituted IMF.
11. Evaluation of the ability of IMF containing maltodextrin to support diarrhoeal enterotoxin production by *B. cereus II* isolates.
12. Studies on the efficacy of current infant bottle cleaning and sterilisation techniques to reduce or eliminate the microbial load of contaminated infant feeding bottles.
13. Studies on the antagonistic process between *L. monocytogenes* and *Bacillus spp.*, the latter organisms being isolated from reconstituted IMF.

14. Studies on the antagonistic process between other bacteria (including *L. monocytogenes*) and these *Bacillus spp*.
15. Investigation of the nature of the antagonistic activity produced by the *Bacillus* isolates against *L. monocytogenes*.

2 Literature review

2.1 An overview of the genus *Listeria*

The following section provides a general overview to the genus *Listeria*, making particular reference to the species *Listeria monocytogenes*. In order to satisfy this requirement, the following aspects were covered in some detail, taxonomy and classification of the genus *Listeria*, a physical description of the *Listeria* species, with particular emphasis placed on the thermal resistance capability of *L. monocytogenes*, the natural reservoirs of this micro-organism, the pathogenicity and clinical implications of the infection "listeriosis", outbreaks of this disease where food was shown to be the vehicle of transmission, the methods for the detection of *Listeria* spp. in foods, the measures currently employed to control either contamination and/or subsequent proliferation of *L. monocytogenes* in food products, the existence and cultural properties of morphological variants of *L. monocytogenes*, and finally, the antagonistic interaction between certain microorganisms and their metabolites (e.g. Lactic acid bacteria and bacteriocins) and *L. monocytogenes*.

2.1.1 Taxonomy of the Genus *Listeria*

Listeria monocytogenes was first described by Murray *et al.* (1926) as a small rod shaped bacterium, and named the micro-organism *Bacterium monocytogenes* because of a characteristic "monocytosis" found in the infected blood of laboratory rabbits and guinea pigs.

Pirie *et al.* (1927) isolated an organism, from the liver of infected gerbils, similar to that described by the previous authors and named it *Listerella hepatolytica* in honour of Lord Lister. The bacterium was further described as the causative agent of "circling disease" (an infection now known as listeriosis) in sheep by Gill in 1933. *Bacterium monocytogenes* was first isolated from infected humans, presenting symptoms of mononucleosis (Nyfeldt, 1929), and from infants with systemic infections in 1929 (Burn, 1936) and 1934 (Potel, 1953) respectively.

When the common identity of *B. monocytogenes* and *L. hepatolytica* was established, the name *Listerella monocytogenes* was initially proposed, but later rejected as this generic name already existed (i.e. it was applied to a group of slime moulds). However, Pirie (1940) suggested that the organism be named *Listeria monocytogenes*, which was later accepted.

The genus *Listeria* was initially composed of one species (*monocytogenes*) and it was classified for a time in Bergey's Manual of Determinative Bacteriology in the family *Corynebacteriaceae*. However, in the more recent 9th edition of Bergey's Manual, the genus *Listeria* is listed along with *Lactobacillus, Erysipelothrix* and *Brochothrix* and other genera in the section entitled "Regular, Non- Sporing, Gram Positive Rods" (Seeliger and Jones, 1986). Indeed, both the intra and intergeneric taxonomy of bacteria belonging to the genus *Listeria* have been problematic for a number of years.

Listeria monocytogenes was the only recognised species within the genus until 1961; *Listeria denitrificans*, *Listeria grayii* and *Listeria murrayi* were added to the genus in 1961, 1966 and 1971 respectively (Seeliger and Jones, 1986). In 1984, all serovar 5 strains of *L. monocytogenes* exhibiting a strong β-haemolytic reaction were proposed as a separate species, *Listeria ivanovii* (Farber and Peterkin, 1991). Furthermore, non-pathogenic strains of *L. monocytogenes* belonging to the serovar 6 were recognised as a new species, *Listeria innocua* (Seeliger and Jones, 1986). *Listeria welshimeri* and *L. seeligeri* were added to the genus in 1983 (Farber and Peterkin, 1991).

However, after further DNA base composition and DNA-DNA hybridization studies, it was concluded that *L. denitrificans* should be transferred to a new genus *Jonesia*, as *J. denitrificans* (Rocourt *et al.*, 1987). It was further proposed that *L. grayii* and *L. murrayi* be reclassified as a new genus *Murraya*, as *M. grayii* and *M. grayii subsp. murrayi* respectively (Stuart and Welshimer, 1974) and that the species *L. ivanovii* be subdivided into the genomic groups, *Listeria ivanovii subsp. ivanovii* and *Listeria ivanovii subsp. indoniesis subsp. nov* (Boerlin *et al.*, 1992).

According to Hartford and Sneath (1993), the genus *Listeria* currently consists of 5 species: *L. monocytogenes, L. ivanovii subsp. ivanovii, L. ivanovii subsp. indoniesis, L. innocua, L. seeligeri* and *L. welshimeri*.

2.1.2 Morphological and cultural characteristics of *Listeria* species

Seeliger and Jones (1986) described young cells of *Listeria* species as regular short rods, 0.4-0.5 μm in diameter and 0.5-2.0 μm in length with rounded ends. These cells can occur singly, in pairs, in short chains and/or they maybe arranged at an angle to each other to give a V shaped appearance.

Young and older *Listeria* cells (3-5 days) exhibit a Gram positive and variable reaction respectively and the cells do not form either endospores or capsules. *Listeria* cells are designated as non-acid fast (Seeliger and Jones, 1986). *Listeria* are peritrichously flagellated and exhibit motility in a characteristic tumbling or slightly rotating fashion. Motility is best demonstrated in nonselective liquid or broth media at 20 to 25°C. Furthermore, the degree to which flagella are produced is temperature related, higher temperatures suppress the expression of motility by markedly reducing the production of flagella. Most *Listeria* cultures grown below 37°C will still demonstrate some movement (Lovett 1989).

Listeria species are considered both aerobic and facultative anaerobic (Seeliger and Jones, 1986). Surface colonies of *Listeria* (24 to 48 hours old) grown on nutrient agar are 0.5-1.5 mm in diameter, round, translucent with a dew drop appearance, low convex with finely textured surface and an entire edge. According to Burn (1935), Seeliger and Jones (1986) and Batista (1993), the colony morphology of *Listeria* may vary on an agar surface, giving rise to different pleomorphic surface culture forms which can be designated as smooth (S), intermediate (I) or rough (R). Indeed, the surface cutures of *Listeria monocytogenes* appear bluish grey by normal illumination and characteristically blue green by Henry's oblique transmitted light (Seeliger and Jones, 1986; Henry, 1933; Lachica, 1990). A fuller description of *Listeria* colonial dissociation is described in section 2.1.10.

Listeria grows well on Blood agar and some species express a β-haemolytic reaction. *Listeria ivanovii* is strongly haemolytic and exhibits a very wide zone of haemolysis, whereas *L. monocytogenes* and *L. seeligeri* produce a narrower zone (which sometimes may only be observed by removing the colony). The other members of the

Listeria genus (i.e. *L. innocua, L. welshimeri, L. grayii* and *L. murrayi*) are non-haemolytic (Lovett, 1989, Batista, 1993).

While *L. monocytogenes* has an optimum growth temperature range between 30 and 37°C, it has an overall growth range between 1 and 45°C (Seeliger and Jones, 1986, Lovett 1989). However, further reports revealed that this psychrotrophic microorganism may be able to grow as low as -0.4°C (Walker *et al.* 1990) and as high as 50°C (Farber and Peterkin, 1991). Indeed, it is well recognised that *Listeria* can tolerate and grow at commercial refrigeration temperatures with a generation time of approximately 1.5 divisions per day (Donnelly and Briggs, 1986, Seeliger and Jones, 1986, Ryser and Marth, 1987, Rosenow and Marth, 1987, Brackett, 1988, Ferguson and Shelef, 1990). Research had further proven that this psychrotrophic microorganism can withstand repeated freezing and thawing (Bryan, 1969, Seeliger and Jones, 1986, Palumbo and Williams, 1989).

Members of the genus *Listeria* can grow in nutrient broth supplemented with 10% NaCl (Seeliger and Jones 1986), while Marth (1993) revealed that the organism can tolerate up to 12% NaCl. According to the work of Petran and Zottola (1989), *Listeria* can grow between pH 4.7 and 9.2 (George *et al.*, 1988). Indeed, the minimum pH at which *Listeria* growth occurs (and is sustained) depends on the temperature of cultivation and the concentration and type of organic acid employed in the growth medium (Sorrels *et al.*, 1989, Parish and Higgins, 1989, Ahamad and Marth, 1989, Buchanan and Golden, 1994). Moreover, *L. monocytogenes* appears to tolerate lower pH values at refrigeration temperatures compared to growth at ambient temperatures (Parish and Higgins, 1989).

While the optimum water activity (a_w) level for *Listeria* growth is approximately 0.97 a_w, survival and growth at aw values as low as 0.86 and 0.79 have been reported in the literature (Johnson *et al.* 1988). Indeed, it has been established that *L. monocytogenes* can not only survive spray drying (In't Veld *et al.*, 1991), but it can persist in dried milk powder for up to 16 weeks (Doyle *et al.* 1985 and Prentice 1989).

2.1.3 Thermal Resistance of *Listeria monocytogenes*

With reports that *L. monocytogenes* is potentially present in raw and pasteurised milk (Gitter *et al.*, 1980, Fleming *et al.*, 1985, Lovett *et al.*, 1987, Fenlon and Wilson, 1988, Fenlon *et al.*, 1995), that it has the capablity of growth in food at refrigeration temperatures (Donnelly and Briggs, 1986, Doyle *et al.*, 1987, Rosenow and Marth, 1987) and knowing that the organism is a potentially lethal human pathogen (Gellin and Broome, 1989, Lovett, 1989, Farber and Peterkin, 1991, Jacquet *et al.*, 1995), considerable emphasis has been placed on efficacy of commercial pasteurisation to completely eliminate this organism from milk.

Indeed, infection of the cow's udder with *L. monocytogenes*, either due to the consumption of improperly fermented silage and/or from other environmental sources may lead to the shedding of this potentially pathogenic organism at populations of 10^3 ml^{-1} or greater in milk (Barza, 1985, Doyle *et al.*, 1987, Bahk and Marth, 1990).

Conflicting reports have appeared on the ability of *L. monocytogenes* to survive the minimum High Temperature Short Time Pasteurisation (HTST) processing (71.7°C for 15 secs) as outlined by the Food and Drug Administration (Food and Drug Administration, 1983). Bearns and Girard (1958) were the first to report that *L. monocytogenes* cells could survive pasteurisation in milk; however, Donnelly *et al.* (1987) determined that the heat inactivation method employed by these authors could have resulted in an over estimation of the thermotolerance of the organism.

Although some reports have appeared regarding the ability of this psychrotrophic bacterium (in naturally or artificially contaminated raw milk) to survive the holder (i.e. 63°C for 30 mins) and the HTST pasteurisation processes (Fernandez Garayzabel *et al.*, 1986, Fernandez Garayzabel *et al.*, 1987, Doyle *et al.*, 1987, Knabel *et al.*, 1990 and Farber, *et al.*, 1992), numerous studies support the efficacy of both thermal processes to eliminate *L. monocytogenes* cells from milk (Bradshaw *et al.*, 1985, Bunning *et al.*, 1986, 1988, 1992, Donnelly and Briggs, 1986, Donnelly *et al.*, 1987, Beckers *et al.*, 1987 and Golden *et al.*, 1988). Indeed, many inter-related factors have been shown to influence heat resistance of non-spore forming microorganisms

(Hansen and Riemann, 1963, Busta, 1978), including the time of exposure, physiological age, the cell density during injury, the composition of the injury menstruum and recovery medium, the diluent, the plating medium etc.

Fleming *et al.*, (1985) and Doyle *et al.*, (1987), postulated that the ability of this potentially pathogenic organism to survive pasteurisation could be due to the thermal protective effect provided by an intracellular location (i.e. within bovine polymorphonuclear leukocytes-PMLs), as invasive *L. monocytogenes* cells can invade these PMLs as part of their infection cycle. However, other reports have demonstrated that this intracellular location within bovine phagocytes did not significantly enhance this organisms thermotolerance (Bunning *et al.*, 1986, 1988, 1992). Furthermore, Dickerson (1986) dismissed this intracellular shielding effect simply on the known thermodynamics of the pasteurisation equipment design.

Most thermotolerance studies have utilised these *Listeria* cells grown at or below 37°C (Bearns and Girard, 1956, Bradshaw *et al.*, 1985, 1987, Bunning *et al.*, 1986, 1988, 1992, Donnelly *et al.*, 1986, 1987, Golden *et al.*, 1988). However, the normal bovine body temperature is approximately 39°C, but this may rise to as high as 42.8°C as a result of a severe listeriosis infection, creating a condition of heat stress for the growing *Listeria* cells (Castle and Watkins, 1984, Knabel *et al.*, 1990). A number of reports reveal that cultivation of *Listeria* cells at the elevated temperature of 43°C prior to HTST pasteurisation permits low level survival of *L. monocytogenes*, where these cells were subsequently enriched under strictly anaerobic conditions (Knabel *et al.*, 1990, Farber *et al.*, 1992).

Moreover, it has been documented that certain Gram negative (Elliker and Frazier, 1938) and Gram positive bacteria (White, 1953, Jenkins *et al.*, 1988) cultivated at above normal physiological growth temperatures, exhibit greater thermal resistance. Beuchat (1978) speculated that growth at higher temperatures resulted in the production of thermostable membranes, which result in increased thermotolerance.

Another popular theory which this research favours, is that the cultivation of *Listeria* cells at above their optimum growth temperatures for brief temperatures induces the synthesis of heat shock proteins (HSPS), which can partially protect the *Listeria* cells against subsequent exposure to normally lethal temperatures (Golden *et al.*, 1988,

Linton *et al.*, 1990, Busch and Donnelly, 1992, Meyer and Donnelly, 1992). Moreover, the rate of HSP synthesis was shown to increase 20 fold in *E. coli*, which had been shifted from an incubation temperature of 30 to 42°C.

The use of strict anaerobic recovery techniques following the heat stressing of *Listeria* cells can lead to the resuscitation of significantly more cells than compared to recovery of damaged cells in the presence of oxygen (Knabel *et al.*, 1990, Farber *et al.*, 1992). For example, a decimal reduction time value (D value) at 62.8°C for cells grown at 43°C and recovered anaerobically were at least 6 fold greater that those determined previously by using cells grown at 37°C and enumerated aerobically (Knabel *et al.*, 1990). It is postulated that the oxygen sensitivity of heat stressed *L. monocytogenes* may be due to the inactivation of certain enzymes during heating, such as catalase and superoxide dismutase (Knabel *et al.*, 1990).

There is growing concern in the food industry regarding the organism's thermotolerance in addition to the ability of stressed cells to resuscitate at refrigeration temperature, as this may lead to the development of clinically significant pathogenic strains. Indeed, Northolt *et al.*, (1988) found that the number of *Listeria* cells recovered increased 2 to 34 times, if the cells were allowed to resuscitate at 4°C before incubation at 37°C.

A number of reports suggest that *Listeria's* heat resistance capability can vary according to the particular strain of *L. monocytogenes* employed. Golden *et al.*, (1988), illustrated that one strain, Brie 1, was significantly more heat resistant compared to other strains, however, the authors believed that it was unlikely that this strain would survive pasteurising in freely suspended milk. Subsequent research by Sorquist (1993, 1994) also demonstrated a variation in thermotolerance among 12 serotypes of *L. monocytogenes* at 62°C. It is well established that a greater concentration of heat stressed *Listeria* cells can repair their injury and subsequently form colonies on non-selective as opposed to selective plating media, which are supplemented with either antibiotics, metal ions and/or dyes (Buchanan *et al.*, 1988, Fedio and Jackson, 1989, Linton *et al.*, 1990 and Schoeni *et al.*, 1991).

Linton *et al.* (1990) also showed that *Listeria* cells heat treated in their stationary phase of growth are more heat tolerant compared to cells which have been treated in

the exponential phase of growth. Finally, current work by Lou (1995) suggests that *L. monocytogenes* cells might be able to survive certain 'hurdle' effects (e.g. acidity, low moisture conditions, sub-lethal heating, etc.) if they are not encountered all at once. Lou's findings indicate that cells that have survived a small hurdle first might be strong enough to "leap" over the subsequent larger hurdles.

2.1.4 Reservoirs of *Listeria species.*

Humans, animals and the environment serve as reservoirs of *L. monocytogenes.* This microorganism has been isolated from a wide variety of animals, including sheep, cattle, goats, pig, horses, geese, gulls, ducks, pigeon, turkeys, chickens, partridge, eagle, parrot, canary, owl, rat, moose, fox, lemming, rabbit, ferret, hare, guinea pig, chinchilla, skunk, mink, dog, cat, deer, racoon and mouse (Brackett, 1988).

The finding of this organism in humans without symptoms may be the result of colonisation of the intestinal tract (Lovett, 1989). Indeed, it is likely that healthy animals and humans may potentially act as asymptomatic carriers of *L. monocytogenes* (Ralovich, 1987, Lovett, 1989, Farber and Peterkin, 1991), where it is excreted into the environment. According to Farber and Peterkin (1991), between 5 and 10% of the human population carry *L. monocytogenes* in their faeces. Thus, due to this high rate of clinically healthy carriers, the presence of *L. monocytogenes* in the faeces is not necessarily an indication of infection. Furthermore, the prevalence of *Listeria* carriage on the hands of food workers was investigated by Kerr *et al.* (1993), where the authors discovered that 12% of these workers carried *Listeria* spp. and 7% carried *L. monocytogenes.*

Listeria monocytogenes is now known to be ubiquitously distributed in the environment (Prentice and Neaves, 1988, Lovett, 1989). Until about 1960 however, listeriosis was regarded as a contagious disease of domestic animals (Seeliger, 1961) and most isolations of this organism were made from infected animals.

The first of many environmental isolations was made by Gray (1960), where this author demonstrated the presence of *Listeria* in poorly made silage.

The introduction of enrichment techniques allowed the isolation of this potential pathogen from a wide variety of food and environmental sources, including raw and pasteurised milk (Flemming *et al.*, 1985, Fernandez Garayzabal *et al.*, 1988, Rea *et al.*, 1992, Fenlon *et al.*, 1995), cheese (Fraser and McKain, 1995), poultry (Prentice and Neaves, 1988, Farber *et al.*, 1989, Lovett, 1989), raw delicatessen products (Jacquet *et al.*, 1995), vegetables (Farber *et al.*, 1989, Schlech III *et al.*, 1983), meat and meat products (WHO report, 1988) and dried milk powders (Doyle *et al.*, 1985). Environmental sources of this organism include, sewage and surface water (Kamplemacher and van Noorle Jansen, 1980), straw , sewage sludge and river water (Prentice and Neaves, 1988). Indeed the organism has been isolated from silage that was implicated as the food source for several cases of proven animal *Listeriosis* (Rea *et al.*, 1992). *Listeria monocytogenes* has also been isolated from food processing and domestic environments (Cox *et al.*, 1989). These authors revealed that 20% of the household refrigerators were contaminated with this organism.

2.1.5 Pathogenesis of *Listeria* species.

The genus *Listeria* has been shown to consist of both human and animal bacterial pathogens, such as *L. monocytogenes, L. ivanovii* and *L. seeligeri*. While *L. monocytogenes* is responsible for the majority of human and animal *Listeriosis; L. seeligeri* and *L. ivanovii* were previously documented as the infectious agent in one and three cases of human *Listeriosis* respectively (Gellin and Broome, 1989, Farber and Peterkin, 1991).

There are 16 serotypes of *L. monocytogenes* which have been identified by the serological grouping of 5 heat labile flagella antigens and 14 carbohydrate containing heat stable antigens, of which serotypes 1/2a, 1/2b, and 4b have been responsible for more than 90% of human disease (Gellin and Broome, 1989).

Listeria monocytogenes, belongs to the facultative intracellular bacteria which can invade, survive and replicate within phagocytic cells such as macrophages and monocytes (Gaillard *et al.*, 1987, Farber and Peterkin, 1991). After uptake into the phagosome *L. monocytogenes* escape via the action of an SH-activated cytolytic

toxin, listerolysin. This haemolysin is required for intracellular survival (Gaillard *et al.*, 1987). In the cytoplasm. the bacteria replicate, become coated with actin filaments and spread, intracellularly and from cell to cell, by forming a tail of polymerised actin (Mounier *et al.*, 1990). Other less well defined virulence factors include phosphatidylinositol specific phospholipase C, a metalloprotease, a lecithinase, and ACT-A, a gene product involved in actin polymerisation (Portnoy *et al.*, 1992).

Although the portal of entry for virulent *L. monocytogenes* may include an ocular and/or dermal routes (i.e. via direct contact), the primary route of infection is considered to via be the intestine. Experiments with animals have revealed that orally inoculated *L. monocytogenes* cells are rapidly taken up by epithelial cells at the tips of the ileal villi and progress to the villus stroma, where they are take-up by macrophages (Rosenow and Marth, 1987, Gellin and Broome, 1989).

Rosenow and Marth (1987) reported that as few as 10^2-10^3 cells may be sufficient to initiate an infection and this infection level may vary with host susceptibility (WHO, 1982). Infection with *L. monocytogenes* can cause several different forms of listeriosis which can be divided into 5 categories, namely: pregnancy infections, granulomatosis infantiseptica (e.g. in early onset perinates), sepsis (e.g. in late onset perinates), meningoencephalitis (e.g. in the elderly and/or immunocompromised) and focal infections (e.g. laboratory workers and veterinarians) (Gellin and Broome, 1989, Bahk and Marth, 1990).

Listeriosis during pregnancy may occur any time, but is most frequently documented in the third trimester. The patient may first experience only a' flulike illness, with fever, backpain, headache, discoloured urine and myalgia. Associated gastrointestinal symptoms including diarrhoea and abdominal cramps are less common. This prodrome, which occurs in approximately two thirds of cases (Boucher and Yonekura, 1986) represents a bacteremic period and *L. monocytogenes* can be isolated from the blood, umbilical cord blood, lochia, vaginal mucus, urine and placental tissue. This prodrome may progress to amnioitis (i.e. infection of the fetus either via the transplacental route or during delivery), and result in premature delivery or septic abortion within 3 to 7 days for the pregnant woman. The infection is usually

self-limiting because the nidis of the infection is eliminated with the birth of the infected foetus and delivery of the intrauterine contents (Gellin and Broome 1989).

Perinatal listeriosis is rarely life threatening to the mother, but transplacental infection of the foetus is likely to produce bacteremia, and neonatal morbidity and mortality are common, with fatality rates ranging from 3 to 50% in neonates born alive (Boucher and Yonekura 1986).

Two clinical forms of neonatal listeriosis have been described, i.e. early and late onset. Early onset neonatal listeriosis, occurs in infants infected in utero, most often to infants of mothers who experienced bacteremic, flulike prodrome before the onset of labour. Indeed, bacteremia produced in the foetus can lead to the presence of *L. monocytogenes* in foetal urine. The foetal urine is discharged in the amniotic fluid, which is then aspirated by the foetus leading to wide spread involvement of the respiratory tract and gastrointestinal tract. The manifestations of the early infection are apparent either at birth, within a few hours of birth or within a few days of life, and symptoms include, respiratory distress, heart failure, difficult and forced respiration, cyanosis, refusal to drink, vomiting, convulsions and mucus in the stools (Gellin and Broome, 1989).

Moreover, listeriosis of the new-born is characterised by involvement of the new-born organs, which develop nodules or granulomata; including the liver, spleen, adrenal gland, lung, oesophagus, posterior pharyngeal wall and tonsils (Bahk and Marth, 1990).

Late onset neonatal listeriosis usually affects full term infants of mothers who have had uncomplicated pregnancy. The infants are usually healthy at birth, and manifestations of the infections are apparent several days after birth, where symptoms are more likely to be of a meningitis nature as opposed to sepsis. (Gellin and Broome, 1989).

In non-pregnant adults and children, listeriosis is principally an infection of cancer patients, transplant recipients, persons receiving immunosuppressive therapy and elderly persons (Barza, 1985, Lovett *et al.*, 1989, Bahk and Marth, 1990). Other disorders associated with an increase in susceptibility to listeriosis include diabetes, alcoholic and non-alcoholic liver disease, chronic renal disease, and patients suffering

form an underlying viral infection such as HIV (Barza, 1985, Gellin and Broome, 1989, Alterkruse *et al.*, 1994).

Up to 30% of adults and 50% of children and young adults who have listeriosis have no apparent compromising condition (Ciesielski *et al.*, 1988, Farber and Peterkin, 1991).

The most common manifestation of listeriosis to occur in new-born and aged persons is meningitis, while less common infections of the central nervous system (CNS) include, meningoencephalitis, cerebritis, brain stem abscesses and spinal cord abscesses (Gellin and Broome, 1989). The clinical course is fulminant, and the fatality rate in untreated patients or those treated is approximately 70% (Lovett, 1989).

Despite reports which suggest that listeriosis is restricted to a sensitive minority of the community (Cox, 1989), Archer (1989) reported that cancer patients, persons with renal disease, diabetics (11 million), organ transplant recipients (70,000), individuals with liver disease, AIDS patients, the elderly, pregnant women (3.8 million), perinates and neonates comprised 32% of listeriosis cases in 1986 and together these groups comprised a significant proportion of the US population.

Thus, *L. monocytogenes* has a striking propensity to affect humans which have either an under developed immune system (e.g. neonates) and/or adults who are either immunocompromised and/or pregnant (Gellin and Broome, 1989, Lovett, 1989, Farber and Peterkin, 1991). Treatment of listeriosis patients is normally through the administration of a wide spectrum of antibiotics (Lovett, 1989).

2.1.6 Outbreaks of listeriosis

While the zoonotic nature of listeriosis was reported as early as 1911 in infected sheep, sporadic outbreaks of human listeriosis were not recorded until 1929 and 1936 in adults and infants respectively (Bulletin of the WHO, 1988). Indeed, the listeriosis vehicle of infection was unknown until the late 1970s, direct transmission from infected animals to farm workers and/or veterinarians was well documented (Farber and Peterkin, 1991).

With the improvements in microbiological enrichment and detection techniques, the direct link between virulent strains of *L. monocytogenes* and infected foods was demonstrated, such as, raw vegetables and coleslaw, pasteurised milk, soft cheese, paté, jellied pork tongue (Lovett, 1989, Jacquet *et al.*, 1995). It is now believed that the main route of infection is foodborne, where *L. monocytogenes* cells are ingested via contaminated food and subsequently cause disease (listeriosis) in susceptible individuals. A review of the reported foodborne cases of listeriosis for England, Wales and Scotland from 1990 to 1994 is illustrated in Table 2.1, where *L. monocytogenes* was implicated as the aetiological agent. Although the number of *Listeriosis* cases is significantly small compared to the total number of actual foodborne illness cases, it would be considered foolhardy to dismiss this pathogen on the basis of this alone, i.e. owing to the organism's detrimental effect on vulnerable people.

The global impact of human listeriosis is illustrated in Table 2.2. The reports from an extensive literature review reveals a world-wide recurrence of listeriosis, having appeared in an almost regular pattern throughout the 1980's, when in 1992, the largest foodborne outbreak of listeriosis occurred to date in France, where 85 people died as a result of consumption of contaminated jellied pork tongue (Jacquet *et al.*, 1995).

The recent decline in international foodborne outbreaks of listeriosis has been attributed to the susceptible consumer groups adhering to medical advice and avoiding the consumption of high risks foods, such as soft cheeses and pate; microbiological screening for the organism in commerce, adequate reheating of chilled foods, virulence characteristics of the organism and other less well defined reasons (McLaughlan 1991).

Table 2.1 Foodborne infection and/or intoxication cases in England, Wales and Scotland, where *Bacillus* spp. and *L. monocytogenes* were implicated as the aetiological agents.

Year	Total number of cases (all micro-organisms)[c]		Total number of cases (*L. monocytogenes*)		Total number of cases (*Bacillus spp.*)	
	England/ Wales[a]	Scotland[c]	England/ Wales	Scotland	England/ Wales	Scotland
1990	36,945	1,419	116	15	162	6
1991	35,384	1,057	127	12	95	8
1992	42,550	780	106	15	182	0
1993	44,271	1,092	102	15	31	0
1994	50,692	987	112	12	87	2

Source: [a] Communicable Disease Surveillance Centre, 61 Colindale Avenue, London
[b] Communicable Disease (Scotland) Unit, Ruchill Hospital, Glasgow, Scotland.
[c] The food-borne illness cases in England/Wales and Scotland are illustrated separately.

Table 2.2 Incidence of human listeriosis world-wide

Location	Year	Number of cases (Number of deaths)	Vehicle of infection
Boston, USA[c]	1979	20 (5)	Lettuce, raw vegetables
New Zealand[c]	1980	29 (9)	Seafood, shellfish, raw fish
Canada[b]	1981	41 (17)	Coleslaw
Massachusetts, USA[c]	1983	49 (4)	Pasteurised milk
Canton de Vaud, Switzerland[c]	1983-87	122 (31)	Vacherin Mont' dor cheese
California, USA[a]	1985	142 (48)	Jaksco Mexican cheese
Linz, Austria[c]	1986	20	Raw milk, vegetables
Philadelphia, USA[c]	1986-1987	36 (16)	Ice cream, salami
Los Angeles, USA[c]	1987	11	Butter
Engalnd and Wales[e]	1986	1	Imported soft cheese
Yugoslavia[d]	1988	1	Human breast milk
Connecticut, USA[c]	1989	9 (1)	Shrimp
Western Australia[c]	1990	16 (11)	Paté
France[a]	1992	279 (85)	Pork tongue

[a] Jacquet *et al.* 1995 [b] Gillan and Broome, 1986 [c] Farber and Peterkin, 1991
[d] Svabic-Vlahovic *et al.* 1988 [e] Lund *et al.* 1991

2.1.7 Methods and media for the isolation and enumeration of *Listeria monocytogenes*.

Interest in *L. monocytogenes* as a food transmitted bacterial pathogen has increased steadily since the 1980s, indeed direct and indirect epidemiological links between the consumption of coleslaw, pasteurised milk, soft cheese and jellied pork tongue and outbreaks of human listeriosis have led to intense research, especially in the area of enrichment and identification methodologies (e.g. the recovery of non-heat and heat injured *L. monocytogenes* from mixed culture microflora food samples) (Jacquet *et al.*, 1995).

Although Golden *et al.* (1988) determined that differential plating procedures could successfully be utilised for the recovery of healthy and injured *L. monocytogenes* cells from foods containing low populations of competing microflora, other research has demonstrated that these direct plating procedures will not recover low numbers of either injured or non-injured *Listeria* cells from foods which have been heavily contaminated with a mixture of microflora. As a result, one and two stage selective enrichment techniques have been therefore developed (Bailey *et al.*, 1990a, 1990b).

The psychrotrophic nature of *L. monocytogenes* has led to the development of the cold enrichment technique (Gray *et al.*, 1948), where a sample of contaminated food is transferred to either a non-selective or selective broth and subsequently incubated for 24 days at refrigeration temperature (4°C). While this enrichment method has improved the isolation rate of *L. monocytogenes* from heavily contaminated foods, the extended storage time required by this procedure makes it totally impractical for routine microbiological analysis of foods.

The addition of antibiotics (moxalactam, ceftaxidime, polymyxin, nalidixic acid etc.), dyes (acriflavine), metal compounds and other chemicals (lithium chloride, phenylethanol, glycine, potassium tellurite) to enrichment broth's and/or plating media has allowed the use of elevated storage temperatures and as a result, the incubation times required for the subsequent isolation of *Listeria* has been reduced to 1 to 2 days (Cassiday and Brackett, 1989, Patel and Beuchat, 1995). Of these selective agents employed for the subsequent isolation of *L. monocytogenes* from foods containing

mixed microflora, cyclohexamide (fungicidal agent) and acriflavine (suppresses Gram positive bacteria) are favoured by the majority of researchers.

While numerous enrichment procedures have been developed (Rodriguez *et al.*, 1984, Doyle and Schoeni, 1986, Hayes *et al.*, 1986, Lovett *et al.*, 1987, McClain and Lee, 1988), the two most recently used methods for the detection and recovery of *L. monocytogenes* cells from contaminated foods , are the United States Food and Drug Administration-(FDA) and United States Department of Agriculture-Food Safety and Inspection Service-(USDA-FSIS) methods (Lovett, 1988, McClain and Lee, 1989). These USDA-FSIS and the FDA standard methods are recommended for the isolation of *L. monocytogenes* cells from meat/poultry and milk/dairy products respectively.

Listeria enrichment broth (LEB) which is the primary selective enrichment broth used in the FDA isolation technique, consists of trypticase soy broth and yeast extract, which has been supplemented with nalidixic acid, acriflavine and cycloheximide (Lovett, 1988). This FDA method initially involved streaking the undiluted and diluted LEB culture (1:10 in 0.5%KOH) onto modified McBride Agar (MMB) after 1 and 7 days incubation at 30°C. However, further revisions to this FDA method were proposed by several researchers to make the procedure more practical, namely: a reduction in the enrichment period from 7 to 2 days, eliminating the KOH pre-treatment and the addition of lithium chloride-phenylethanol moxalactam agar (LPM) as a second selective plating medium (Lovett and Hitchins, 1988, Batista 1993).

The USDA-FSIS procedure has undergone dramatic changes since its development by McClain and Lee in 1988. Originally, the procedure consisted of a two stage enrichment in which University of Vermont broth (UVM) was employed at both the primary and secondary enrichment stages (Donnelly and Baignet, 1986), Where the secondary broth (UVM II) differed from the primary selective broth (UVMI) simply by containing twice the amount of acriflavine. The enrichment broths were subsequently streaked onto LPM agar.

Further changes to this USDA-FSIS method were made by McClain and Lee in 1989, such as; the secondary enrichment broth UVMII was replaced by Frazer broth (i.e. UVM I to which lithium chloride and ferric ammonium citrate were added by Frazer and Sperber in 1988); the LPM agar was replaced by modified Oxford agar (i.e.

Oxford agar from which acriflavine, cycloheximide, fosfomycin, cefotetan and 50% of the colistin sulphate were omitted and moxalactam was added) as the sole plating medium. The authors demonstrated that the hydrolysis of esculin and subsequent interaction of ferric ammonium citrate produced a characteristic blackening of the Frazer broth, in addition, the appearance of this black halo around presumptive *Listeria* colonies permits the presumptive identification of *Listeria* spp. in a relatively short time period.

The efficacy of the USDA and FDA procedures (i.e. to detect non heat and heat injured *L. monocytogenes* cells in contaminated foods) were compared by several researchers. While Westoo and Peterz (1992) and Hayes *et al.*, (1992) did not find any significant difference between these methods, the latter authors revealed that recovery of *Listeria* cells was significantly better when 2 enrichment methods were used in combination, compared to using only one method.

While the current USDA protocol for the detection of *L. monocytogenes* indicates streaking form Frazer broth regardless of colour reaction after 26 hours incubation, the protocol allows testing to be terminated for samples in which the Frazer broth does not blacken. Kornacki *et al.* (1993) demonstrated that of the samples which had blackened in Frazer broth after 48 hours enrichment, 3.3% of these did not contain any *Listeria*. There is currently, no approved official method for determining low levels of viable *L. monocytogenes* in milk and milk products (Twedt *et al.*, 1994).

Among the numerous researchers that compared the efficacy of various *L. monocytogenes* enrichment procedures, only a few (Crawford *et al.*, 1989, Bailey *et al.*, 1990a, McCarthy *et al.*, 1990, Lovett *et al.*, 1991, Warburton *et al.*, 1992 and Patel and Beuchat, 1995) studied the recovery of heat damaged *Listeria* cells from food.

The potential for food poisoning caused by contaminated food products containing heat damaged, but subsequently repaired microorganisms is very real (Smith and Palumbo 1982, Mossel 1989). Indeed, microbial metabolic injury often results in their inability to recover and form colonies on selective media, that otherwise would have supported growth. Most media currently used for the enrichment of *Listeria* spp. (from food samples) contain inhibitory substances which prevents repair and

subsequent colony formation. The presence of phenylethanol, acriflavine, NaCl and polymyxin-acriflavine were all found to be detrimental to the recovery of heat -injured *L. monocytogenes* cells (Smith and Archer, 1988, Crawford *et al.*, 1989, Leasor *et al.*., 1990, Warburton *et al.*, 1991).

McCarthy (1990) demonstrated no significant difference between the FDA and USDA detection methods were shrimp had been artificially inoculated with heat treated *L. monocytogenes* cells, while the work of Bailey *et al.*, 1990a showed that LEB was an inferior enrichment medium to that of UVMI for recovering *L. monocytogenes* from soft cheese and chicken post heat treatment.

Warburton *et al.* (1991) and Bailey *et al.* (1990a, 1990b) illustrated that the enrichment period of the FDA method could be shortened from 7 to 2 days without substantially reducing the number of positive samples. These authors also demonstrated that Frazer broth could be a very useful tool for screening food samples for *L. monocytogenes* cells (but needed to be more selective due to the high incidence of false positives).

Warburton *et al.*, (1992) carried out a comparative study on modified versions of the FDA (Lovett and Hitchins, 1988) and USDA-FSIS (McClain and Lee, 1989) methods for food and environmental samples containing both non-heat and heat stressed *L. monocytogenes* cells. Both procedures were modified by the inclusion of additional plating media, and the replacement of both secondary enrichment broths with modified Frazer broth (which contained lower levels of acriflavine- McClain and Lee, 1989). The modified versions of the FDA and USDA methods incorporated a primary and secondary enrichment stage where the broth cultures were incubated for 24 and 48 hours at 30°C prior to streaking onto a variety of selective plating media. While both enrichment methods were comparable in the recovery of low numbers of heat treated *Listeria* cells, Oxford agar was revealed to be a superior recovery medium compared to LPM, Modified Oxford Agar (MOX) and Palcam Agar.

Patel and Beuchat, (1995) compared the efficacy of LEB, UVM, MUVM, and Frazer broth (FB) to recover heat damaged *L. monocytogenes* cells. The authors observed either no change or a decrease in cell number after 8 hours. The recovery of heat stressed cells were higher in LEB , MUVM, UVM, and FB respectively . In addition

the supplementation of the enrichment media with catalase enhanced the recovery of heat stressed *L. monocytogenes* cells. Moreover, the recovery of sublethally injured *Listeria* cells has been improved by the incorporating of various chemicals (e.g. catalase) into enrichment media (Knabel *et al.*, 1990, Busch and Donnelly, 1992, Martin and Katz, 1993).

2.1.8 Methods for the detection of *Listeria monocytogenes*.

Regarding a food poisoning outbreak (i.e. in order to make the link between the ingestion of the incriminating food and the disease), it is essential that epidemiological rather than circumstantial evidence is obtained. As certain food products have only a short shelf life, it is imperative that satisfactory food quality monitoring is routinely carried out and that subsequent microbiological results are obtained quickly. Conventional *Listeria* culture methodology may take up to 5-6 days simply to confirm absence of the organism (Ninet *et al.*, 1992).

In response to this shortcoming , rapid diagnostic or identification techniques, such as, flow cytometry (Donnelly and Baignet, 1988), polyclonal or monoclonal antibodied enzyme-linked immunosorbent assays or "ELISA" techniques (Beumer *et al.*, 1988, Norrung *et al.*, 1991, Oladepo *et al.*,1992), DNA hybridization probes (Klinger *et al.*, 1988, Notermann *et al.*, 1989, Ninet *et al.*, 1992, Url *et al.*, 1993), polymerase chain reaction probes (Wernars *et al.*, 1991), impedimetric assays (Phillips and Griffiths, 1989, Hancock *et al.*, 1993) and biochemical and/or sugar fermentation galleries (Robinson and Cunningham, 1991, Bannerman *et al.*, 1992, Bille *et al.*, 1992, Kerr *et al.*, 1992) have recently developed to specifically detect *Listeria* spp. and in some cases, more precisely *L. monocytogenes*.

At the present time, *Listeria* isolates are mainly recognised on the basis of morphological and biochemical characteristics (Bille and Doyle, 1991). The ultimate aim of a commercial diagnostic test kit, is to quickly detect low numbers of either non-stressed or stressed *Listeria* cells in a heavily contaminated food product (containing a heterogeneous microbial population), at an economical cost and using

minimum labour and to eliminate or reduce the initial pre-enrichment stages (i.e. to presumptively detect *L. monocytogenes* in a food sample in less than one day).

The Gene-trak (Url *et al.*, 1993) and the Accuprobe (Ninet *et al.*, 1992) are commercially available DNA probes, which can seek out and specifically bind to target *L. monocytogenes* DNA which has a complimentary nucleotide sequence. In order to function effectively, both probes require an initial enrichment stage, in addition to obtaining well isolated single colonies of *L. monocytogenes* on an agar medium. Indeed, a number of technical problems arise for the direct detection of this organism (and/or other pathogenic bacteria) in food samples via DNA probes, namely: levels of *L. monocytogenes* in food samples maybe very low relative to indigenous flora (e.g. current DNA probes have a minimum detection limit of approximately 10^5 organisms ml^{-1}), and the pathogens may be non-uniformly distributed in the food product (therefore, making it extremely difficult for the probes to seek out individual microorganisms); the background foodstuffs currently give high spectrophotometrical readings in the absence of *L. monocytogenes* (Eley, 1990).

The ELISA techniques also require an enrichment state, Beumer *et al.*, (1988) demonstrated that their procedure required a 48 hour pre-enrichment where the *Listeria* cells had reached a threshold level of approximately 10^6 CFU ml^{-1}, without competitors. In addition, a time consuming antigen extraction stage is often required (Eley, 1990).

Hancock *et al.* (1993) demonstrated rapid detection of *Listeria* spp. via a selective impedimetric assay. This technique detected *L. monocytogenes*, *L. innocua* and *L. welshimeri* in foods but could not differentiate between these *Listeria spp.*

In relation to recent innovations in the area of *Listeria* strain identification, a number of commercial *Listeria* identification kits (which are designed to replace the time consuming and labour intensive conventional culture procedures) are available. Bannerman *et al.*. (1992) evaluated the MICRO-ID *Listeria* system (Organon Teknika), which utilises reagents impregnated paper disks that react with either bacterial enzymes or metabolic products to produce a readily identifiable colour changes. The authors found that 98.8% (409/414) strains of *Listeria* challenged , were identified correctly by this miniaturised biochemical /fermentation test kit (this

biochemical test kit was run in conjunction with the complimentary tests, CAMP test and β-Haemolysis). The 10 strip API *Listeria* test kit (Biomerieux) was evaluated by Bille *et al.* (1992), where they illustrated that 97% (252/258) of the test strains were correctly identified by the system. Therefore, the use of MICRO-ID *Listeria* in combination with API *Listeria* biochemical test system should permit the identification of presumptive *Listeria* colonies to species level (in approximately 98% of cases). Indeed, these test kits are easy to operate and interpret, in addition to allowing genus identification and species determination in 24 hours.

2.1.9 Control of *Listeria* species in food

Not everyone that comes in contact with *L. monocytogenes* becomes ill. Indeed, in healthy individuals this potentially deadly pathogen is cleared rapidly by the liver, and to a lesser degree by the spleen. (Farber and Peterkin, 1991). Moreover, the cell mediated immune response is stimulated and macrophages and polymorphonuclear leukocytes are sensitised (Barza, 1985, Bahk and Marth, 1990). For those that do become ill, prompt diagnosis of the disease and subsequent administration of therapeutic drugs is recommended.

A considerable amount of attention has recently focused on the ability to detect and control this pathogen in food products. Farber (1993) has suggested that a multifaceted line of approach should be considered to control *L. monocytogenes* in foodstuffs and that the general consumer population should come to terms with the fact that this organism may never be totally eliminated in foods due its ubiquitous presence in the environment. However, in terms of a multifaceted approach to controlling the proliferation of this bacterium, the author suggest that 4 main factors should be addressed , namely: (1) regulatory monitoring of high risk and other ready to eat food products which are capable of supporting the growth of *Listeria* spp., (2) use of the hazard analysis critical control point (HACCP) approach for controlling the growth and spread of *L. monocytogenes* in food products and through the factory, (3) use of barriers or "hurdles" to control the development of and/or to inactivate *Listeria*

in foods and (4) educate the high risk consumer groups regarding these high risk food groups.

Regarding the use of hurdles, there has been a considerable amount of research carried out on the potential use of bacteriocin producing starter cultures or the direct addition of bacteriocins to foods, in order to help control the proliferation of *Listeria* (Parente and Hill, 1992).

Installation and proper validation of total quality management (e.g. based on ISO9000 standards) to the manufacture, distribution and sale of food products should help control the risk of potential contamination of food with this micro-organism. Indeed, the microbiological quality of the food product should not be left to the Quality Control Department, but should be seen as the combined responsibly of each department in company, the distributer , the vendor and most importantly the consumer (Mettler, 1989).

2.1.10 Morphological variants of *Listeria monocytogenes.*

Many researchers have reported the existence of different cell and colony forms of *L. monocytogenes*, which these authors designated as either smooth, intermediate or rough (Burn, 1935, Webb and Barber, 1937, Barber, 1939, Paterson 1940, Nyfeldt, 1953, Gray *et al.* 1957, Seeliger 1961, Kathariou *et al.*, 1987, Kuhn and Goebel, 1989, Bubert *et al.*, 1992 and Batista, 1993).

According to these authors, a typical smooth 48 hour surface colony form of *L. monocytogenes* is circular, domed to convex centres with entire edges, 1 to 1.5 mm in diameter. They are transparent by transmitted light, milky white by reflected light, butyrous in consistency and easily emulsified in saline. Seeliger and Jones (1986) described the smooth cell phenotype of *L. monocytogenes* as, short non-spore forming rods (0.4-0.5 μm in diameter and 0.5-2.0 μm in length), with young cultures consisting predominantly of coccoid cells, although slightly curved rods were occasionally observed which appeared singly, in short chains of 3-8 cells (which were sometimes arranged in a V or Y shape).

Rough forms (R-forms) may or may not show differences from the parent strain or from each other in either size, texture, consistency, configuration, opacity, colour, morphology, biochemical reaction, degree of haemolysis and virulence (Seeliger, 1961). Indeed, Anton (1934) thought the R-form of *L. monocytogenes* to be a fungus, due to the presence of long filaments.

Webb and Barber (1937) described the rough phenotype to be slightly larger than the S-form, with a domed centre, but the periphery margin was described as flattened, spreading, wrinkled and undulated. The R-form of Barber (1939) was slightly larger (1.5-2.0 μm in diameter) and more flattened; with a matt surface, umbonate centre and fimbriate edge gradually spreading outwards until a colony size of 5 mm is reached in 5 days at room temperature. Gray *et al.* (1957) reported the rough type to be relatively large, erose to lobate outer edge with a coarsely textured surface.

The rough mutant colony forms in early cultures (7-12 hours at 37°C) were reported to consist almost entirely of long non-spore forming filaments averaging about 60 μm in length, while at 48 hours growth, rods from 1-50 μm predominate (short filaments from 10-12 μm are present in moderate numbers). Several researchers reported the predominance or presence of *L. monocytogenes* in the form of long filaments (i.e. isolated from the rough surface culture form) and highlighted the close resemblance to that of *Erysipelothrix insidosa* (Anton, 1934, Barber, 1939, Nyfeldt, 1953, Seeliger, 1961).

A 60 KDa extracellular protein (termed p60) is an important house keeping protein for virulent strains of *L. monocytogenes* (Bubert *et al.* 1992, Wuenscher *et al.* 1993, Kuhn and Goebel, 1989). Culture variants of *L. monocytogenes* which produce a reduced level of this p60 protein, exhibit a rough colony morphology and form long cell chains with unseparated septa between the individual bacterial cells (Wuenscher *et al.*, 1993). Moreover, this rough morphological phenotype apparently correlates with the amount of p60 secreted into the supernatant by these R-colony form mutants. In the studies carried out by Wuenscher *et al.* (1993), the secretion of small amounts of p60 into the supernatant by a *L. monocytogenes* rough variant (RIII) during its exponential growth phase, resulted in the formation of long cell chains. However,

when this p60 protein accumulated in the supernatant (in the stationary growth phase), a considerable shortening of the cell chains was observed.

While Bubert *et al.* (1992) demonstrated that the addition of partially purified p60 to rough mutant forms causes the cell chains to desegregate, which results in the formation of single cells. The gene encoding p60 (designated "iap" for invasion associated protein) was recently cloned and sequenced, and Wuenscher *et al.* (1993) believe that the expression of the p60 protein in *L. monocytogenes* causes the disruption of the cell chains and is regulated at the post-transcriptional level. These authors suggest that p60 maybe a murein hydrolase, the purpose of which is to degrade its own cell wall. Therefore, by this definition p60 could be classified as a listerial autolysin, which functions include, wall growth, turnover and splitting of septa on cell separation.

While there is controversy surrounding the virulence capability of pleomorphic colony forms of *L. monocytogenes*, the majority of researchers demonstrated that the R-form exhibits reduced virulence and do not effectively invade 3T6 mouse fibroblasts (Potel and Schulze-Lammers, 1985, Kuhn *et al.*, 1989). While Webb and Barber, (1937) failed to find any variation in virulence between smooth and rough colony forms. Hunter *et al.*, (1950) demonstrated that 5 colony variants of *L. monocytogenes* exhibited a decreased pathogenicity for the chicken embryo and the white mouse.

Another area of considerable disagreement concerns the reversion of the rough colony form back to its parent smooth form. Seeliger and Linzenmeir (1953) failed to find evidence of R to S form reversion, while Burn, (1935) and Potel, (1963) illustrated either partial or complete reversion.

2.1.11 Antagonistic interactions between *L. monocytogenes* and other microorganisms.

The realisation that foods can play a major role in the transmission of *L. monocytogenes* has led to interest in new methods for controlling this psychrotrophic pathogen, particularly in refrigerated foods. Among the techniques being investigated, the use of antagonistic bacteria or bacteriocins produced by these micro-

organisms has received increased attention over the past number of years (Gouet *et al.*, 1978, Ortel, 1989, Hoover *et al.*, 1989, Carminati *et al.*, 1989, Benkerroum and Sandine, 1988, Spelhaug and Harlander, 1989, Ahn and Stiles, 1990, Lewus *et al.*, 1991, Schillinger *et al.*, 1991, Berry *et al.*, 1991, Marrug, 1991, Buchanan and Klawitter, 1992, Parente and Hill, 1992, Bankerroum *et al.*, 1993, Rekhif *et al.*, 1994, Bower *et al.*, 1995).

The Lactic acid bacteria (represented by Lactococci, Streptococci, Lactobacilli, Pediococci and Leuconostoc) produce a number of antimicrobial agents including metabolic products such as organic acids, hydrogen peroxide, lacterperoxidase, diacetyl and other more specifically inhibitory substances such as antibiotics and bacteriocins (Spelhaug and Harlander ,1989, Marugg, 1991). Marugg (1991) revealed that the *Lactic acid bacteria* produce two classes of antimicrobial proteins known as "bacteriocins", those bacteriocins that are cidal to a narrow range of target organisms, usually closely related to the producer strain and those (e.g. Nisin from *Lactococcus lactis* and Pediocin A from *Pediococcus pentosaceus*) that inhibit a broad spectrum of Gram positive bacteria. The latter type of bacteriocin inhibits the development of many species and strains of spoilage and pathogenic microorganisms associated with foods including *Clostridiium botulinum* and *L. monocytogenes* (Spelhaug and Harlander, 1989, Bower *et al.*, 1995). Jack *et al.* (1995) described the bacteriocins of Gram positive bacteria as ribosomally synthesised, cationic, low molecular weight and heat stable.

Indeed, it seems likely that most if not all bacteria are capable of producing a heterogenous array of molecultes in the course of their growth that may be inhibitory to either themselves or other organisms (Jack *et al.*, 1995). These molecules include: (1) toxins (e.g. diptheria, tetanus and cholera toxins with actions against eukaryotic cells, (2) bacteriolytic enzymes such as lysostaphin, (3) bacteriophages, (4) by-products of primary metabolic pathways such as organic acids, ammonia and hydrogen peroxide, (5) antibiotic substances like gramicidin and (6) bacteriocins (Jack *et al.*, 1995)

To date, a number of bacteriocin producing microorganisms have been identified which exhibit an inhibitory or bacteriocidal effect on the development of *L.*

monocytogenes cells, namely *Streptococcus lactis, Streptococcus cremoris, Streptococcus lactis subsp. diacetylactis, Lactococcus lactis, Leuconostoc mesenteroides subsp. mesenteroides, Lactobacillus curvatus, Lactobacillus plantarum, Enterococcus faecium, Pediococcus pentosaceous, Pediococcus acidilactici, Carnobacterium piscola* and *Bacillus amyloliquefaciens* (Spelhaug and Harlander, 1989, Carminati *et al.*, 1989, Marugg, 1991 and Batista, (1993). Indeed, the employment of bacteriocins has been demonstrated to be particularly useful in controlling the growth of foodborne pathogens (including *L. monocytogenes*) in dairy and meat products (Gouet *et al.*, 1978, Pucci *et al.*, 1988, Chung *et al.*, 1989, Beliard *et al.*, 1989, Carminati *et al.*, 1989, Berry *et al.*, 1990, Nielsen *et al.*, 1990, Schillinger *et al.*, 1991, Buchanan and Klawitter, 1991, Marugg, 1991).

Moreover, bacteriocins may offer safe and more natural means of controlling undesirable bacterial contaminants, particularly pathogenic to man and the application of these bacteriocidal proteins in combination with traditional methods of preservation, in addition to proper hygienic processing could be effective in controlling spoilage and pathogenic bacteria in foods (Marugg, 1991).

However, while the use of bacteriocins seems promising, Rekhif *et al.*, (1994) did reveal the existence of a mutant strain of *L. monocytogenes* (ATTC 1513) which exhibited resistance to the inhibitory action of 3 bacteriocins produced by *Lactic acid bacteria* (e.g. mesenterocin 5C, curvaticin 13 and plantaricin C19).

2.2 An overview of the genus *Bacillus*

The following section provides a general overview of the genus *Bacillus*. Indeed, during the course of this review, the following topics will be addressed in some detail, namely: the difficulty and controversy surrounding *Bacillus* taxonomy and subsequent identification to species level, the diverse morphological, biochemical and physiological properties of the genus, the ecology of the organisms with particular emphasis placed on the inherent ability of these aerobic spore forming bacteria to tolerate adverse environmental conditions (e.g. drying and heating), pathogenicity of *Bacillus*, and the association with several opportunist infections and foodborne illness

outbreaks (i.e. an epidemiology study), and finally, the preventative measures currently employed to control the development of *Bacillus* spp. in food.

2.2.1 Taxonomy of the genus *Bacillus*.

The genus *Bacillus* is one of 13 bacterial genera which can form endospores. The endospore-formers are currently separated into different genera by virtue of the fact that they differ morphologically, physiologically, and genetically from one another (Berkeley and Ali, 1994).

Before 1946, classification of the genus *Bacillus* could best be described as chaotic. Indeed, attempts made to identify aerobic sporeforming bacteria to species level had only left the taxonomy of this large group of bacteria in a state of total confusion (Smith *et al.*, 1946, Goepfert *et al.*, 1972). In 1946 and 1952, Smith *et al.* published 2 excellent monographs in which a rational and orderly approach to the taxonomy of *Bacillus* were addressed. Moreover, the 1952 revision "USDA Monograph No.16" became and remains the principal authority on the taxonomy of the genus and it is the basis for separation and identification of *Bacillus* today,

Contributions from other researchers, such as: Wolf and Barker, 1968, Gordan *et al.*, 1973, Bonde, 1975, Boeye and Aerts, 1976, Logan and Berkeley, 1981, 1984, Sneath, 1986, Priest and Alexander, 1988, Parry *et al.*, 1988, Gordan *et al.*, 1989, Ash, 1991, and Berkeley and Ali, 1994 should not go unappreciated.

The genus *Bacillus* embraces a very large number of bacteria with a great diversity of properties; proposals have been put forward to simplify the genus by dividing it into several genera, but each proposal has had its complications and the *Bacillus* group has remained in tact.

Smith *et al.* (1952) carried out an extremely careful and thorough investigation in which 1134 mesophilic endospore forming *Bacillus* strains consisting of 19 species, were arranged into 3 groups based on morphology (i.e. the shape of the spore and the swelling or absence of swelling of the sporangium by the spore) and physiology. The authors recognised, however, that these morphological/physiological groups did not

represent ordered, distinct compartments, but rather that, with each group, there existed a spectrum of properties (Parry *et al.*, 1988).

Morphological group one comprises *Bacillus* that possess ellipsoidal or cylindrical shaped endospores which do not distend the sporangium. Further subdivision of group one was made on the basis of vegetative cell size and the presence or absence of vacuoles within the vegetative cells which are grown on glucose nutrient agar. Large vacuolated cell species (>1.0 μm) include *B. megaterium* and members of the *B. cereus group* (i.e. *B. cereus, B. cereus var mycoides, B, cereus var anthracis* and *B. thuringiensis*), while smaller non-vacuolated cell species (<1.0 um) include members of the *B. subtilis group* (i.e., *B. subtilis, B. licheniformis* and *B. pumilus), B. coagulans* and *B. firmus*.

The spores of *B. coagulans* may or may not swell the sporangium and for this reason, this bacterium is considered as an intermediate between this first morphological grouping and morphological group 2 . The decision to consider *B. mycoides. B. anthracis* and *B. thuringiensis* as variables of *B. cereus* (rather than separate spp,) has invoked favourable response and criticism (Goepfert *et al.*, 1972).

Morphological group 2 consists of *B. polymyxa, B. macerans, B. circulans, B. stearothermophilus, B. alvei, B. laterosporus, B. brevis, B. pulvifaciens* and *B. popillae*, the sporangium of each of these species is swollen by the presence of an oval endospore.

Bacillus sphaericus is assigned to morphological group 3, this grouping is defined on the basis of spherical or round spores in a swollen sporangium. Members of the genus *Bacillus* which do not exhibit morphological and/or cultural characteristics representative of these 3 morphological groupings are unclassfied and described as "*incertae sedis*".

Several numerical studies by Bonde, (1975), Logan and Berkeley, (1981), Priest *et al.*, (1981) and Priest and Alexander (1988), as well as other molecular work (Fox *et al.*, 1977) provided a good measure of agreement with this taxonomic arrangement. However, these subsequent taxonomic studies all indicate the probable existence of more than 3 groups of species. The genus *Bacillus* currently resides in "Endospore-

forming Gram positive rods and cocci" as described in section 13 of volume 2 of Bergey's Manual (Sneath 1986).

Today, the classification of this genus is still unsatisfactory, as many researchers have demonstrated close relationships to non-spore formers, in addition to a heterogeneity of many of its species as well as genetically (which now has a % DNA base composition range of 36-69%). A generally accepted classification rule states that bacteria differing by more than 10 mol% G+C (%GC) in their base composition should not be considered as members of the same genus (Bull *et al.*, 1992, Berkeley and Ali, 1994). Indeed, the situation has not been improved by the addition of many new species since the publication of the approved list of bacterial names (Sherman *et al.*, 1980). At that time the approved list contained 31 spp., by the end of 1992, the number had increased to 67 (Berkeley and Ali, 1994).

Ash *et al.* (1991) in their molecular systematic study, examined the sequence of the 16s rRNA of 51 *Bacillus spp.* and found the results of their work indicated the existence of 5 phylogenetically distinct clusters. There is some measure of agreement between these and the groupings which employed earlier phenotypic numerical studies (Smith *et al.*, 1952, Gordan *et al.*, 1973, Logan and Berkeley, 1981).

The International Committee on Systematic Bacteriology -Subcommittee on the taxonomy of the genus *Bacillus* (1995), have recently stated that amplified ribosomal DNA restriction analysis (i.e. molecular systematic work), pyrolysis mass spectrometry and API tests (supplemented with microscopic data) have revealed similar taxonomic structures, in which established *Bacillus* spp. have been well separated. Further information relating to classification of the genus *Bacillus* and identification to species level is provided in section 5.1.

2.2.2 Methods employed for identification of *Bacillus* to species level.

Until quite recently, clinical isolates of *Bacillus* were not identified further than "aerobic spore forming bacteria (ASB)" because they were deemed to be of no clinical significance. Indeed, the fact that identification of the presumptive *Bacillus* spp. to species level was considered difficult and tedious did not improve the situation, Over

the last decade or so, attention has focused on the possible role of these inconsequential ASBs in opportunist infections (i.e. where the absence of known human pathogens have been confirmed).

As a result of this heightened interest and the need for better differentiation between members of the genus *Bacillus*, many researchers have set out to make identification of *Bacillus* less arduous and more comprehensive by characterising the species on the basis of a relatively small number of morphological, physiological and/or biochemical tests (Gordan *et al.*, 1983, Parry *et al.*, 1988, Priest and Alexander, 1988). Indeed, Logan and Berkeley, (1981) and a review by the International Committee on Systematic Bacteriology -Subcommittee on the taxonomy of the genus *Bacillus* (1995), have evaluated the efficacy of two commercially available API BioMerieux products, API 50 CHB and API 20 E [i.e. the *Bacillus* identification protocol only employs the first 12 biochemical tests in this 20 E *(Enterobacteriaciae)* test system] biochemical galleries and the authors have demonstrated that these test kits do successfully assist identification down to *Bacillus* species level. Further work by Bryant and his colleagues (1985) has confirmed that *Bacillus* spp. may be successfully differentiated via probabilistic identification methods based on API tests.

Other techniques employed to characterise the *Bacillus spp.* include: serotyping and phagetyping (de Barjac, 1981 and Kawano *et al.*, 1981), protein electrophoresis (Owens *et al.*, 1984, Zahner *et al.*, 1994), pyrolysis mass spectrometry (Berkeley *et al.*, 1992), pryolysis gas-liquid chromatography (O'Donnell *et al.*, 1981) and molecular methods such as amplified polymorphic DNA techniques (Stephan *et al.*, 1994). A colour atlas for the identification of *Bacillus* species is currently available (Parry *et al.*, 1988).

Two *Bacillus cereus* diarrhoeal enterotoxin detection test kits are commercially available. Becker and Wong (1994) evaluated the *Bacillus cereus* enterotoxin reverse phase latex agglutination (RPLA) test kit (Oxoid product) and the *Bacillus* diarrhoeal enterotoxin visual immunoassay (Tecra product) and revealed that the former method successfully identifies the L2 component of the haemolysin BL (known to cause fluid accumulation in the rabbit ileal loop test), while the latter test system apparently detects 2 non toxic proteins.

Further work by Granum (1993) demonstrated a good correlation between standard techniques to detect enterotoxin production (i.e. Western Immunoblot technique and the vascular permeability reaction) and the Oxoid RPLA enterotoxin identification test system.

The author stated that the RPLA test system was a simple and reliable method, suitable for routine laboratory testing. Buchanan and Schultz (1992) also established a general agreement between the detection of the heat labile enterotoxin produced by *Bacillus* using the RPLA test system and the production of a cytotoxic response in HEP2 and CHO cell lines.

There is currently no available simple test system for the identification of the extracellular factor produced by *B. cereus* thought to cause the emetic type food poisoning syndrome.

2.2.3 General morphological and physiological characteristics of *Bacillus.*

The cells of *Bacillus* species are rod shaped, straight or nearly so, and may occur singly or in chains (which may be of considerable length). The rods may have rounded or square ends and vary in size from quite small (0.5-1.2 μm) to rather large (2.5 to 10 μm). The cytoplasm may be vacuolated or may stain uniformly and certain *Bacillus* strains may contain intracellular parasporal bodies or protein crystals (Sneath 1986).

Bacillus cells exhibit a positive Gram stain reaction, or Gram positive reactions only in the early stages of growth. Motility (where evident) is by means of peritrichous flagellation. Oxygen is the terminal electron acceptor, replaced in some species by alternatives. While most *Bacillus* spp. are unpigmented, some species do produce pigmentation (e.g. yellow, black, orange, brown etc.) on certain media (Sneath, 1986).

Members of the genus *Bacillus* exhibit a wide physiological ability; namely , psychrotrophic (i.e. growth at 4°C, e.g. *B. globisporus, B. cereus* etc.) or thermophilic (i.e. growth at 65°C, e.g. *B. stearothermophilus*), acidophilic (i.e.

growth at pH below 5.7, e.g. *B. subtilis*) or alkaphilic (i.e. growth at pH above 8, e.g. *B. brevis*); some strains are salt tolerant (i.e. growth in 7.5% NaCl, e.g. *B. licheniformis*) while others are not (i.e. *B. lentimorbus* does not grow in 2% NaCl) (Sneath, 1986, Parry *et al.*, 1988).

Catalase, although formed by the majority of *Bacillus species* (e.g. except certain strains of *B. stearothermophilus*) is either positive or negative. The members of the genus *Bacillus* comprise of chemoorganotrophs, prototrophs and auxotrophs. Capsules are synthesised (e.g. *B. anthracis*) under certain condition in some species (Sneath, 1986).

The appearance of surface colonies of *Bacillus* strains vary considerably depending on environmental factors. These include factors that are imposed on the test culture (e.g. composition of the growth medium, temperature of incubation, water activity level etc.) as well as those resulting from changes in the environment caused by the development of the culture (Collins *et al.*, 1989). A description of the colonies of *Bacillus* species on blood agar has been given by Parry *et al.*, (1988). Although different strains of a particular *Bacillus* species often form colonies which appear similar enough to be characteristic, colonial variants of most species have been described. Indeed, some extreme variations in colony morphology can be observed among members of the *B. subtilis group*.

Endospores are formed by all members of the genus *Bacillus* (not more than one per cell), which are very resistant to many adverse conditions. Endospores are very resistant to many adverse environmental conditions, including elevated temperatures (whereas heating above 70°C for 10 mins or longer will destroy vegetative cells of almost all known non-spore forming bacteria, the endospores of *Bacillus spp.* survive this treatment), dehydration (e.g. within soil samples, air drying will destroy most bacteria while spore-formers like *Bacillus* may survive drying for long periods), destruction by disinfecting chemicals (e.g. a study has shown that spores of *Bacillus* strains survive exposure to 50% (v/v) ethanol for at least one hour, while vegetative cells are normally killed) (Sneath, 1986).

2.2.4 Life cycle of *Bacillus* species

Members of the genus *Bacillus* are potentially able to form resting cells in the stationary phase of growth (or if vegetative cells are transferred from a rich to a poor culture medium). Resting cells are formed intracellularly and are therefore designated as "endospores". These spores are known to possess different properties to those of vegetative cells: namely, optical refractivity, ultra structure, chemical composition and resistance to chemical and physical stresses (i.e. which leads to the vegetative cell being killed).

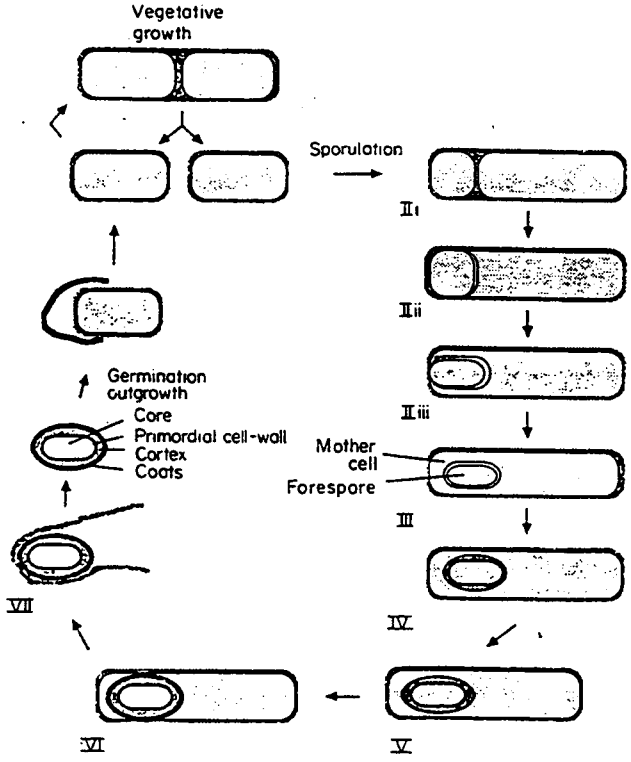
The formation of endospores is a multiphase process which is similar in all *Bacillus* strains so far studied (illustrated in Figure 2.1): During vegetative growth, cells divide by binary fission. Under conditions of nutrient deprivation asymmetric septation takes place (stage IIi). The forespore is then engulfed by the mother cell membrane (stage IIii and IIiii) and become surrounded by mother cell cytoplasm (Stage III). During stage IV, the primordial cell wall is laid down surrounding the forespore inner membrane (black line) and then the cortex (shaded area) on the outside of the primordial cell wall. The coats (black line) begin to be deposited outside the cortex during stage V. The spore matures (stage VI) and is released on lysis of the mother cell (stage VII). Germination of the endospores occurs in the presence of germinants and the cortex is hydrolysed, the germinated spore can then outgrew to form a new vegetative cell (Foster, 1994).

Germination of endospores leads to an important phase in the life of each member of the genus *Bacillus*, as the ability of this microorganism to survive harsh environmental conditions such as heating, dehydration etc., is of practical concern to those seeking to achieve sterility and/or a high level of microbiological quality in food products.

Although the mechanism for the initiation of sporulation is unknown, once initiated, the formation of endospores is an irreversible process. It occurs normally after the exponential growth phase, when the generation time increases. Factors effecting endospore formation include; temperature of growth (i.e. best optimal conditions 25 to 30°C), pH of the medium (i.e. best at pH 7.0), aeration, the presence of minerals (especially Mn^{2+} and Ca^{2+}), the presence of carbon source (i.e. the glucose

concentration is important because sporulation enzymes are under catabolic repression) or nitrogen compounds (Sneath, 1986, Bergere, 1992).

Endospore germination is the process by which a dormant spore develops into an actively growing vegetative cell and it is a stage of great significance for food technologists because the initial contamination of unsterilised milk, dairy products and other non sterilised foodstuffs by microorganisms is mainly in the form of dormant endospores. Moreover, these spores must germinate before the resulting vegetative cell multiplies, proliferates and/or produces toxins. Germination of endospores normally comprises of, a triggering or an initiation mechanism e.g. heat (i.e. this usually involves the interaction of a specific compound "germinant" with the spore, this irreversibly commits the spore to lose its dormancy), initiation (is the sequence of degradative reactions that occur as a result of this triggering mechanism) and finally outgrowth (which is defined as the development of the new vegetative cell from the germinated spore) (Bergere 1992, Foster 1994).



(Figure adopted from Foster, 1994)

Figure 2.1 Stages in the *Bacillus* sporulation process.

Like other spore-formers, *Bacillus cereus* spores are capable of germinating without preliminary heat treatment or heat activation; however, the rate of germination and the proportion of germinated spores are higher when they have been previously subjected to a sublethal heating, such as 70°C for 30 mins (Hurst and Gould, 1983). While the efficiency of activation depends on environmental conditions during heating (e.g. water activity, composition of the culture medium etc.), bacterial spores can also be activated by γ-radiation (doses <0.4-0.5 Mrad), reducing agents (thioglycolate), oxidising agents (perborate) or simply low pH levels (<pH 3.0) (Hurst and Gould, 1983, Bergere, 1992).

Heating at temperatures from 65 to 95°C for various holding time intervals, activates *B. cereus* spores in milk (Stadhouders *et al.*, 1980). Moreover, the so called slow germinating spores of *B. cereus* (which for the main part do not germinate within 24 hours in HTST milk at 20°C) need a more intense heat activation than fast germinating spores (which germinate within 24 hours at 20°C after an equivalent HTST process). Indeed, heating for 2 mins at 65°C brought about a nearly complete germination of the latter spores, which was observed after 24 hours at 20°C, where the former required heating for 2 mins at 85-95°C to obtain the same degree of germination (Stadhouders *et al.*, 1980).

Although certain *Bacillus* endospore producers (e.g. *B. cereus* and *B. megaterium*) respond to a germinant more rapidly and synchronously than others (e.g. *B. subtilis*), the initiation stage in endospore formation, and/or the other events leading to endospore synthesis, is similar for these spp. Indeed, the changes which can occur during germination of *B. cereus* endospores include: (1) loss of heat resistance (2) release of K, then diamimopimelic acid and Ca, (3) loss of refractivity or phase darkening, (4) onset of stainability, (5) release of DPA from the cortex, (6) changes in O.D., fall in extinction, then postgerminative events, (7) increase in energy reactions, (8) increase in permeability (9) increase in spore volume (Bergere, 1992).

As the heat resistance of endospores and/or their germination requirements may significantly vary depending on either the environmental /process conditions (e.g. temperature, pH, a_w, ionic strength, inhibitors) and/or the type of *Bacillus* species

under study, to ensure that every ungerminated spore in the foodstuffs is capable of forming a colony under the assay conditions, the heat treatment must not be too high (as to cause death or stress to the spore) or not too low (when spores need activation). For example, Stadhouders *et al.* (1980) employed a temperature/time regime of 5 mins at 75°C for a heat sensitive strain, and 10 mins at 80°C for the heat stable spore form (which need activation). Germination of *Bacillus* endospores depends on the strains and the germination media, some strains germinated well at 4°C, partly at 2-3°C or even at temperatures near 0°C (Johnson *et al.*, 1983). While the optimum pH for germination varied form 6 to 8 in 10 strains of *B. cereus* (Bergere 1992), spores did not germinate at pH >10 or pH<4.5.

The successive phases in the germination process include: premergent swelling of the spore, emergence and elongation of the new cell. However, if germinated spores are held at conditions that do not support outgrowth, they will autosterilise or become inactive. The rate and extent of *Bacillus* endospore germination in certain milk products (e.g. pasteurised milk, cream) depends on the *Bacillus* strains, heat treatment of milk, and environmental factors. It has been documented that while slow germinating endospores of *B. cereus* are more heat resistant compared to the so called fast germinators, the former require greater heat activation temperatures to germinate. *B. cereus* spores germinate at low temperatures especially in heat treated milk (IDF Bulletin, 1992).

Wilkinson and Davies (1973) reported that activated spores of *B. cereus* germinate more rapidly and more completely in HTST pasteurised milk than in raw milk due to the production of a germination factor (a germinant). At pasteurisation, the germinant produced in milk (molecular weight of 200 Daltons, and identification still unknown), may be formed via 2 processes: one form an interaction between milk constituents alone (i.e. interaction between low and high molecular weight fractions, such as whole casein) and the other involving milk constituents and somatic cells.

2.2.5 Pathogenicity of *Bacillus* species.

Bacillus anthracis is the best known pathogen in the genus, causing a lethal infection known as "anthrax" in infected humans and animals. While anthrax is primarily a disease of herbivorous animals and can be transmitted to humans by direct contact (i.e. from certain animal products), manifesting in the form of intestinal, cutaneous, orthopharyngeal and/or pulmonary anthrax (Logan, 1988).

While Koneman *et al.* (1992) suggested that anthrax is currently of minor significance since it was found that it could be effectively treated in man with penicillin, in many countries this disease remains a major problem. Logan (1988) revealed that anthrax is enzootic in China, Iran, parts of Africa, India and South America, and it is virtually enzootic in many other areas. World-wide there may be as many as 10,000 animal outbreaks each year and, perhaps, 9000 human cases.

Human anthrax infections have been encountered as an occupational disease of veterinarians, agricultural workers, and others who handle animal products (approximately 90% of human cases reported in recent years occurred in mill workers handling imported goat hair) (Koneman *et al.*, 1992). The recent isolation of penicillin resistant strains of *B. anthracis* should, however, serve as a warning that this microorganism should not be treated lightly (Sneath, 1986).

Bacillus cereus, a close relative of *B. anthracis* is undoubtedly the *Bacillus* species of next importance as an opportunist pathogen of man, as well as probably animals (Logan 1988, Granum, 1994). While this organism is best known as the aetiological agent in numerous foodborne intoxications (causing either an emetic and/or a diarrhoeal type syndrome), *B. cereus* has been also implicated as the causative agent in a number of non-food related infections; namely bovine mastitis and haemorrhage, severe systemic and pyogenic infections, gangrene, septic meningitis, cellulitis, panophthalamitis, lung abscesses, endocarditis, bacteremia, septicaemia, pneumonia, empyema, and infant death (Logan, 1988, Koneman *et al.*, 1992). This bacterium has emerged as one of the most virulent and destructive ocular pathogens, responsible for known cases of conjunctivitis, panophthalmitis, keratitis, iridocyclitis, decrayocystitis and orbital abscesses (Logan, 1988).

Through the years it has been the practice of many clinical laboratories to simply discard isolates of *Bacillus spp.* (often designated as "inconsequential aerobic spore forming bacteria"), other than *B. anthracis* or *B. cereus*, as contaminants of skin, hair etc. (Parry *et al.*, 1988, Logan, 1988, Koneman *et al.*, 1992). Several authors have made reference to the fact that in clinical laboratories throughout the world, many *Bacillus spp.* are being discarded daily which were in fact, of 'unappreciated relevance to the infections from which they were isolated' (Gordan *et al.*, 1973, Gilbert *et al.*, 1981, Norris *et al.*, 1981, Gordan *et al.*, 1983).

Indeed, serious opportunist infections associated with significant morbidity and mortality, have been caused by a variety of *Bacillus* spp. (other than *B. cereus*) such as, septicaemia, endocarditis, osteomyelitis, myonectosis stimulating clostridial gas gangrene, necrotizing fascitis, bronchopneumonia, necrotizing pneumonia, empyema, meningitis, peritonis and endophalmitis (Koneman *et al.*, 1992). These life threatening infections have been associated with parenteral drug abusers, various operative procedures, haemodialysis, traumatic wound, burns, immunosuppression and other predisposing factors (Koneman *et al.*, 1992).

These clinically significant *Bacillus spp.* include: *B. subtilis* (causing pneumonia and respiratory diseases), *B. sphaericus* (causing fatal meningitis, bacteremia, endocarditis), *B. megaterium* (causing pharyngitis), *B. pumilus* (causing meningitis and septicaemia), *B. circulans* (causing fatal meningitis), *B. licheniformis* (bacteremia, septicaemia, opthalmitis), *B. mycoides* (meningitis), *B. macerans* (isolated from infected site of melanoma incision), *B. coagulans* (corneal ulceration), *B. thuringiensis* (inflammation and lamphagitis), *B. panotheniticus* (ocular infections), *B. brevis* (corneal ulceration), *B. alvei* (meningitis) and *B. laterosporus* (endophthalmitis) (Parry *et al.*, 1988, Logan, 1988, Koneman *et al.*, 1992).

The increasing frequency with which these opportunist infections are encountered is in part due to host predisposition, by suppressed or compromised immunity, metabolic disorders, malignant disease, exposure by surgical and clinical procedures, accidental trauma and drug abuse (Logan, 1988). With the knowledge that there is an increasing number of *Bacillus* (other than *B. cereus*) bring implicated as the aetiological agent in

food-borne illnesses (section 2.2.6), the detection of these potentially pathogenic organisms from dried infant formulae may be of clinical significance.

2.2.6 Epidemiology and foodborne illness outbreaks associated with *Bacillus* species.

Despite the large number of *Bacillus spp.* implicated in clinically proven opportunist infections, the widespread distribution of *Bacillus* spores in the environment and in foodstuffs, the inherent ability of spores to survive elevated processing temperatures, long term storage in dried food products, and other harsh environmental conditions, only a limited number of *Bacillus spp.* (i.e. *B. cereus, B. subtilis, B. licheniformis, B. pumilus, B. brevis, B. thuringiensis* and *B. sphaericus*) have been implicated as the aetiological agent in proven foodborne illness outbreaks (Kramer and Gilbert, 1989, Granum, 1994, Jackson *et al.*, 1995).

Since the first confirmed report of human food poisoning following the consumption of contaminated vanilla sauce (Hauge 1950), *B. cereus* is now well established as a significant cause of foodborne illness in humans (Table 2.3). Moreover, it is recognised that distinct clinical forms of gastro-enteritis can result from the ingestion of foods heavily contaminated with this aerobic spore former.

The "*B. cereus* diarrhoea type syndrome" is caused by an enterotoxin consisting of one polypeptide chain (mature molecular weight of approximately 40 kDa) and is usually associated with illnesses where proteinaceous foods (e.g. meat, dairy, vegetables, sauces, puddings etc.) have been consumed.

It is characterised by an incubation period of 8-16 hours (average 10-12 hours) prior to the onset of symptoms, such as abdominal pain, profuse watery diarrhoea, rectal tenesmus and occasional nausea that seldom results in vomiting, symptoms are generally resolved within 12-24 hours (Kramer and Gilbert, 1989, Granum, 1994).

The enterotoxin is a cytotoxic (membrane disrupting) protein produced by *B. cereus* during exponential growth and it is considered heat labile (inactivated at 56°C for 5 mins), unstable <pH 4 and susceptible to proteolysis by pepsin, trypsin or chymotrypsin. *Bacillus cereus* can grow quite well anaerobically, and Granum (1994)

demonstrated that it can produce the diarrhoeal enterotoxin at levels comparable to those produced aerobically.

It is unclear if food poisoning is due to preformed enterotoxin or whether *B. cereus* cells are ingested and the enterotoxin is subsequently produced in the small intestine. However, Granum *et al.*, (1993b) demonstrated that it is likely that *B. cereus* foodborne illness is caused by the ingestion of cells or spores rather than the preformed enterotoxin (which is degraded and inactivated on its way to the target).

While the infected dose of *B. cereus* is in the range 10^5-10^8 cells, diarrhoeal enterotoxin is produced before cells reach the 10^7 cells ml^{-1} level. Due to this revelation, Granum *et al.*, (1993b) suggested that the food industry should be concerned about levels as low as 10^3 to 10^4 *B. cereus* cells ml^{-1} or g^{-1} of food.

Furthermore, the level of enterotoxin produced by different strains can vary by a factor of 100 and the enterotoxin has demonstrated a diarrhoegenic activity 100 times greater than the enterotoxin produced by *Clostridium perfringens* (Granum *et al.*, 1993b). Turnbull (1976), demonstrated that this diarrhoeal toxin stimulates the adenylate cyclase cyclic AMP system in intestinal epithelial cells, and probably through this causes fluid accumulation in the ligated ileal section of young rabbits.

Ingestion of cells and spores ($>10^4$ cells ml^{-1}) is the main source of *B. cereus* food poisoning. *B. cereus* was the most common cause of foodborne outbreaks in 1990 for Norway, with the infective dose shown to be approximately 10^4 cells g^{-1} food (Aas *et al.*, 1992). Granum *et al.* (1993b) states that it is possible for some strains of *B. cereus* to colonise the small intestine, at least in some patients, and cause a more severe type of disease.

In contrast, the "*B. cereus* emetic type syndrome" is almost exclusively associated with farinaceous foods, particularly cooked rice, and is characterised by rapid onset (1-5 hours) and common symptoms include, nausea, vomiting, malaise and in some cases followed by diarrhoea (symptoms persist from 6 to 24 hours) (Kramer and Gilbert, 1989). The source of this vomiting type syndrome is still uncertain, Granum (1993a) suggests that it is possibly a lipid of molecular weight (5-7 Kda).

However, unlike the diarrhoeal enterotoxin, this extracellular factor responsible for emetic foodborne illness is extremely stable to heat (i.e. survives 90 mins at 126°C),

pH (stable for 2 hours or more at pH 2) and is resistant to enzymatic degradation by trypsin and pepsin (Christiansson, 1992). Approximately 90% of all emetic syndrome episodes have been associated with the consumption of Cantonese-style cooked rice, while other food vehicles include; vanilla sauce, pasteurised cream, milk puddings, chicken supreme, pasta and reconstituted infant milk formula (Kramer and Gilbert, 1989). These authors further demonstrated an optimal temperature range of 25-30°C for emetic toxin production in rice cultures.

In order to facilitate comparison, *B. cereus* strains producing the emetic or diarrhoeal enterotoxin are designated as *B. cereus I* and *II* respectively (Parry *et al.*, 1988).

Christiansson *et al.* (1989) evaluated a total of 136 strains of *B. cereus II* (isolated from milk) for diarrhoeal toxin production via African green monkey kidney (Hela S3) and human embryonic lung (Hel) cell line cytotoxicity studies. The authors found that 21.8% of isolates exhibited Hel cytotoxicity after 7 hours exposure, which subsequently increased to 73.2% after 24 hours at 30°C.

Furthermore 108 toxin positive strains were acclimatised to grow at 8°C. By cultivating *B. cereus II* strains in brain heart infusion broth (supplemented with either glucose or starch), Garcia-Arribus and Kramer (1991) detected that besides glucose, starch is also a good carbon source for both growth and diarrhoeal toxin production.

While Roy (1993) demonstrated that aeration of *B. cereus II* (in range 10^3 to 10^4 CFU m^{-1}) at low temperatures (e.g. 4°C) appears to encourage diarrhoeal toxin production, contradictory reports by Granum (1993a) reveals that *B. cereus II* produces enterotoxin, haemolysin and lecithinase (phospholipase C) under anaerobic conditions. Other reports supporting the ability of *B. cereus* to grow at refrigeration temperatures include Coghill and Juffs 1979, Griffiths (1990), Van Netten *et al.* 1990 and Buchanan 1992.

While milk and milk products (including dried powders) are major sources of *B. cereus* spores world-wide, surprisingly few reports of *B. cereus* food related illnesses (either the emetic or the diarrhoeal form) have been reported in these food stuffs. Several theories have been proposed for this low incidence of *B. cereus* related food poisoning in milk, namely: insufficient aeration for subsequent toxin production under normal storage conditions; milk has a very low free amino acid content and lacks

glucose (which is a prerequisite for diarrrhoeal toxin production); the number of cells required for toxin production and for subsequent illness to occur is high (e.g. $>10^6$ cells ml^{-1}), which would mean that sweet curdling or organolyptic deterioration would have normally started when the cells are high enough to present a risk; and the virulence characteristic of each *B. cereus* strain (IDF 1992).

Table 2.3 *Bacillus cereus* foodborne illness outbreaks with associated clinical symptoms

Type	Food	Symptoms	Incubation period (hours)	Country
Diarrhoeal type	Cooked rice[1]	CD	2-12	Japan
	Mashed potatoes[1]	CDV	3-10.5	USA
	Chicken dish[1]	CDV	5-10	Canada
	Potatoes, stuffing[1]	CDV	5-14	UK
	Vegetables sprouts[1]	CD	6-15	USA
	Green bean salad[1]	CD	7-15	Canada
	Meat loaf[1]	NCDV	10	USA
	Vanilla sauce[1]	NVD	12-13	Norway
	Chicken pot pie[1]	ND	14	USA
	Barbecue chicken[1]	NCD	15	Canada
	Turkey loaf[1]	CD	?	USA
	Beef stew[2]	CDN	2-13	?
	Home dried apples[2]	CD	8	?
	Human breast milk[3]	D(V)	?	India
	Cream[3]	DV	8-10	UK
	Milk[3]	DC	8-11	Rumania
Intermediate or Not well defined	Chinese food[1]	NVD	2	Canada
	Instant breakfast[1]	NVD	2-3	Canada
	Fried rice[1]	NVD	3-5	Canada
	Malted milk powder[1]	NVD	6	Canada
	Canned tuna[1]	NVD	6	Canada
	Curried chicken[1]	NVD	6	Canada
Emetic type	Packed lunches[1]	V	0.5-3	Japan
	Boiled rice[1]	V	0.5-4	Finland
	Cooked rice[1]	V	0.5-4	Japan, Canada
	Fried rice[1]	DVD	1-6	UK, Canada
	Fried or Boiled rice[1]	V	1.5-4.5	UK
	Vanilla sauce[1]	NV	8	UK
	Risotto beef curry[1]	V	?	UK
	Feta cheese[1]	V	4	Canada
	Skim milk powder[1]	NV	6	Canada
	milk[3]	V	1.5	Denmark

V= vomiting, N=nausea, D= diarrhoea, ( )= mild or few symptoms
Source: [1] Johnson 1984 [2] Kramer and Gilbert 1989 [3] Christiansson 1992

While there is an uncertainty as to the reasons for this low incidence of food poisoning in milk and/or milk products, there is a general agreement among researchers that members of the consumer population which have a predisposing condition (e.g. organ donor recipients) or an underdeveloped immune system (e.g. infants) might be susceptible to lower enterotoxin titres compared to healthy individuals (IDF, 1992).

Beecher and Wong (1994) confirmed that the commercially available *Bacillus cereus* enterotoxin -Reverse Phase Latex Agglutination (RPLA) test kit can be successfully employed to detect the diarrhoegenic enterotoxin produced in milk products. Indeed, Buchanan *et al.* (1992) revealed that there is very good agreement between detection of heat labile enterotoxin production using the RPLA test system and toxin production as analysed via a cytotoxic response in human epidermal carcinoma (Hep-2) and Chinese hamster ovary (CHO) cell lines.

Because *B. cereus* is part of the normal faecal flora, isolation of the microorganism from a patients faeces is not clinically relevant and does not implicate this spp. as a cause of gastrointestinal or invasive illness (Koneman *et al.*, 1992).

Although ideal conditions for establishing the causative role of an organism associated with foodborne illness rarely occur (i.e. it is often difficult to fulfil Koch's postulates), nevertheless, there are documented episodes in which there have been reasonable grounds to implicate *Bacillus spp.* (other than *B. cereus*) in food related illnesses.

Bacillus subtilis has been implicated as the aetiological agent in several foodborne outbreaks where: meat pasties/ pies/ rolls/, Indian or Chinese meat/seafood currie with rice, bread, poultry, pate, pizzas, Greek meat dishes with rice, reconstituted powdered soup and infant milk formula were shown to be the vehicles of infection (Kramer and Gilbert, 1989). Food poisoning symptoms associated with *B. subtilis* were characterised by a notably short incubation period preceded by nausea, vomiting, and stomach cramps, with occasional diarrhoea in about half of the cases (Kramer and Gilbert, 1989).

Other reports have appeared in the literature regarding the isolation of large numbers of *B. licheniformis* (and in the absence of known pathogens) from foods incriminated or associated with outbreaks of food poisoning. Clinical symptoms (e.g. diarrhoea

and sometimes vomiting, incubation period 1-5 hours) and vehicles of transmission (e.g. cooked meat dishes) were similar to that of a *Clostridium perfringens* food related illness (Kramer and Gilbert, 1989).

The intermediate species of the *B. subtilis -licheniformis group, B. pumilus*, has been implicated in a number of food related illness where steak and kidney pie, scotch eggs, canned tomato juice, cheese sandwiches were shown the be the source of infection (Kramer and Gilbert, 1989). Other *Bacillus spp.* implicated in foodborne illnesses include *B. brevis* and *B. sphaericus* (Sneath, 1986, Kramer and Gilbert, 1989, Koneman *et al.*, 1992).

Griffiths (1990) evaluated 83 psychrotrophic *Bacillus spp.* for diarrhoeal toxin production in BHI broth using the RPLA test system, and showed that apart from the 85% of all strains tested exhibiting toxin production, this *B. cereus* enterotoxin test kit system detected toxin production from *B. mycoides, B. thuringiensis, B. circulans, B. lentus, B. pumilus, B. polymyxa , B. carotarum* test cultures, in addition to *B. cereus II.* Further information relating to *Bacillus* food poisoning in England/Wales and Scotland between 1990 and 1994 is provided in Table 2.1.

2.2.7 Ecology of *Bacillus* species.

The inherent ability of *Bacillus* spores to survive harsh environmental conditions, such as heat and drying, the capacity of the vegetative cell form to secrete enzymes capable of degrading many organic materials, and the non-fastidious nature of the organism, facilitate survival and/or growth of this bacterium in a wide variety of environments and food sources. Indeed, both the spore and vegetative morphological form of *Bacillus* have been isolated from a wide range of environments, including soils and clays, sediments, dust, natural waters, vegetation, and from many food types (e.g. cereals and cereal derivatives, milk and milk products, dried foods, spices, meat products and vegetables) (Kim and Goeffert, 1971, Kramer and Griffiths, 1989). As soil samples may contain high concentrations of *Bacillus* endospores, many foodstuffs and environments can be contaminated by *Bacillus spp.* via aerial dust deposition.

A comprehensive study of the incidence of *B. cereus* in food materials has been conducted by Nygren (1962), where the author examined 3,888 food samples and revealed a contamination rate of 51.6% among 1,546 food ingredients, 43.8% of the 1,911 cream and desert dishes and 52.2% of the 431 meat and vegetable products.

The following members of the *Bacillus* genus have been isolated from milk and dried milk products (e,g. infant milk powder): *B. licheniformis, B. cereus, B. circulans, B. firmus, B. pumilus, B. subtilis, B. coagulans, B. sphaericus, B. lentus, B. polymyxa, B. caratarum, B. thuringiensis, B. pumilus* and *B. megaterium* (Veda *et al.* 1980, Singh *et al.* 1980, Ahmed *et al.* 1983, Phillips and Griffiths 1987, Scottish Food Co-ordinating Committee 1987, Burnett 1989, Finoli and Rondini 1989, Crielly *et al.* 1992, Shinagawa 1992 Becker and Terplan 1992, Anderton 1993).

In 1992, Becker and Terplan reported that of 261 samples of infant milk formula (IMF) distributed in 17 countries, 54% were contaminated with *B. cereus* at levels up to 600 g^{-1}, while Veda *et al.* (1976) demonstrated a total aerobic mesophilic count $>10^2$ g^{-1} in 76 dried baby food purchased in Japan.

O'Donovan (1959) found that *B. cereus* contamination of raw milk recovered from creameries or farms were chiefly from cans, after being emptied of milk and subsequently allowed to stand for long periods. Contamination on the farm and/or creameries with *Bacillus* spores has occurred via the udder (either through a mastitic infection or through sticking dirt), the milker, the milking utensils, the surrounding conditions (air), and the dairy factory. It has been demonstrated that even when teats and/or the udder is cleaned, a contamination of small quantities (approximately 20 mg dung) cannot be avoided and counts of 0.2 to 20,000 spores ml^{-1} milk have been recorded (VanHeddeghem and Vlaemyick, 1992). Moreover, soil, dung and fodder contain mainly fast germinating *B. cereus* spores, whereas slow germinating spores are found in the milking equipment, particularly during longer periods at higher outdoor temperatures (Bergere, 1992).

With the elimination of pathogenic, heat labile, non-spore forming microorganisms from milk and other products through the application of bactericidal heating, the thermal processes (e.g. such as pasteurisation) provide favourable conditions for *Bacillus* spore germination (with possible proliferation) (Burnett 1989). Furthermore,

the essential nutrients for the development of most *Bacillus spp.* are provided by milk and/or reconstituted infant milk formulae (IMF). Although milk contains natural antimicrobial systems (e.g. immunoglobulins, lactoferrin (LF) and lactoperoxidase (LP)), both LP and LF are not present in sufficiently high concentration in milk to be totally effective against microbial development. Therefore, natural antimicrobial systems alone cannot be relied upon to eliminate or retard the development of intruding microorganisms into milk (i.e. contaminated milk or IMF may serve as a potential source of pathogenic microorganisms), and that additional heating processes such as sterilisation and pasteurisation are required to control their development (Bergere and Cerf, 1992).

Wilkson and Davies (1973) stated that when milk is intensively heat treated (e.g. a pasteurisation process), it becomes a better growth medium for the subsequent development of germinated cells of *B. cereus* and large numbers of this potential pathogen may be reached in a short time. Indeed, temperatures in excess of 80°C for 15 secs are often required for the activation of slow germinating spores of *B. cereus*.

Bacillus spp. (with particular reference to *B. cereus*) in milk powders are normally present in the form of dormant endospores. The heating procedure applied during the manufacture of medium heat powders is not sufficient to eliminate *B. cereus* spores from this product (Stadhouders 1992). Raju *et al.* (1989) revealed that endospore concentration decreased with increased temperature in milk, however, endospores of *B. cereus* have survived temperatures between 109 and 121°C.

Certain *Bacillus* species have been shown to survive the high sterilisation temperatures employed in the canning industry, causing either swelling, milk coagulation, off flavours, and odours. These spore formers include: *B. subtilis* and *B. megaterium* (mesophiles), *B. coagulans* (facultative thermophile), *B. calidolacticus* and *B. stearothermophilus* (obligate thermophiles) (Crielly *et al.* 1992, 1994, Brown 1994).

Certain members of the genus *Bacillus* are capable of appreciable growth at commercial refrigeration temperatures and the incidents where these species have been recovered from reconstituted infant milk formulae are numerous. Credit *et al.* (1972) demonstrated that *Bacillus spp.* constituted 84% of the microflora of

pasteurised milk stored at 4.5°C over a 30 day observation period. While Coghill and Juffs (1979) revealed that 23.3% of raw milk samples and 16.7% of pasteurised milk were contaminated with aerobic spore formers.

Lovett *et al.* (1979) revealed that *B. circulans* is the most common psychrotroph in raw milk and Griffiths (1990) isolated psychrotrophic spore formers in 70% of pasteurised milk in Scotland (with *B. cereus* being the most common isolate). Reinheimer *et al.* (1987) identified 24 psychrotrophic *Bacillus spp.* in bulk milk from Sante Fe Argentina, where *B. cereus* (48.8%), *B. coagulans* (41.6%) and *B, licheniformis, B. circulans* and *B. brevis* (4%) were the main flora present. Granum *et al.*(1993) demonstrated psychrotrophic abilities among 15% of the 85 *B. cereus* strains isolated from dairy products. Indeed, the same authors showed that 7% of the 85 strains tested were positive for both psychrotrophic growth and, enterotoxin production. Christiansson *et al.* (1989), Griffiths (1989) and Champagne *et al.* (1994) demonstrated the potential for toxigenic *B. cereus* strains to grow at 8°C and /or 6°C.

The faecal carriage of *B. cereus* and other *Bacillus spp.* has been well documented (Kramer and Gilbert, 1989). Indeed, the ingestion of *B. cereus* and other *Bacillus spp.* may be regarded as an inevitable consequence of the wide spread contamination of the food supply. Ghosh (1978) recovered *B. cereus* from 14% of single faecal specimens from 711 adults. While Hoh *et al.* (1982) detected *B. cereus* in 15% of 400 faecal specimens from healthy individuals in Japan.

2.2.8 Control and preventative measures.

The ubiquitous nature of *Bacillus spp.* ensures that these microorganisms will inevitably contaminate a wide range of foods. Therefore, effective control and/or prevention depends on either controlling endospore germination, or the subsequent development of vegetative cells in cooked, and or ready to eat foods.

According to Kramer and Gilbert (1989), cooking methods such as; steaming under pressure, thorough roasting, frying and grilling are most likely to destroy both vegetative cells and heat tolerant spores alike. However, cooking at temperatures at or below 100°C will allow the survival of some *Bacillus* spores.

vegetative cells and heat tolerant spores alike. However, cooking at temperatures at or below 100°C will allow the survival of some *Bacillus* spores.

A significant health concern arises from vegetative cell multiplication during inadequate cooling or non-refrigerated holding of moist, cooked cereal based and/or protein containing food products. With the knowledge that certain cooking procedures provide favourable conditions for the heat activation of *Bacillus* spores, care must be taken to ensure that the foodstuffs are rapidly cooked and adequately stored at a temperature (e.g.≤4°C), which will prevent microbial growth. Food products should always be consumed before their expiry or "best before" date.

Rice prepared in restaurants should be boiled in small quantities on several occasions during the day, thereby reducing the storage time before frying. While *B. cereus* spores germinate at low temperatures, especially in heated milk (Bergere, 1992), Stadhouders *et al.*, (1980) concluded that this property could be employed to prevent the development of the organism by thermising (64-68°C for 10 secs) the milk, with subsequent storage at a suitable temperature which will allow the spores to germinate (e.g. 2 days at 5°C) and finally eliminating the resulting vegetative cells from the product via a pasteurisation treatment (Vlaemynck and Heddeghem 1992). However, not all spores are activated by thermisation (especially slow germinators of *B. cereus*).

2.3 An overview of infant milk formula manufacture, microbiological quality and associated infant health risks.

This final section provides a general overview of infant milk formulae (IMF), as related to its manufacture, microbiological quality and health risks associated with either the proliferation of indigenous microbial flora and/or unhygienic powder reconstitution procedures. Indeed, during the course of this review the following areas were addressed in some detail, namely, the benefits of breast milk and breast feeding, reasons for substituting breast milk for infant milk formulae, the manufacture of IMF and the capability of certain microorganisms to survive the bacteriocidal temperatures employed during the production process, the microbiological quality of infant milk powder and associated foodborne illnesses where IMF was implicated as

the vehicle of transmission, infant health problems associated with improper and unhygienic reconstitution of IMF and finally the improper advertisement, promotion and sale of IMF in under developed countries.

2.3.1 Infant milk formula, a substitute to breast milk.

Until recently, it was thought that the advantages of breast feeding were limited and to most medical and nursing students these advantages comprised of: breast milk being at the right temperature, containing exactly what the baby needs, being bacteria free, coming in cute containers and something that the cat can't get at (Stanway and Stanway, 1983).

However, further work by the scientific community (especially by paediatricians and dieticians) established breast milk to be the perfect food for the nutritional and immunological development of the infant (WHO, 1981, Stanway and Stanway, 1983, Knipschidt, 1986 , Marandi *et al.*, 1993). Indeed, colostrum (i.e. the first milk made by the breast), is rich in protein (9 times richer than mature breast milk), cells, certain amino acids, minerals, vitamins, immunoglobulins (IgA, IgD, IgB_6, IgB_{12}) and has less fat and sugar than mature breast milk.

This immunoglobulin fraction of colostrum plays a large role in the immunological development of the baby, as these antibodies are known to coat the intestinal lining, the result of which help prevent microorganism entering the blood stream, in addition to blocking the absorption of proteins which might set up allergic responses (Stanway and Stanway, 1983). The low fat content of colostrum is advantageous to the new born baby as the infant secretes little lipase of its own and would have difficulty in digesting large amounts of fat in the first few days or so (Stanway and Stanway, 1983). A baby uses only about half the proteins available in cow's milk, whilst a breastfed baby uses all the protein (with virtually no wastage). Indeed, the infant is growing at its fastest in the first 6 months of life, when the protein in breast milk is at its highest (Stanway and Stanway, 1983).

An observation of Table 2.4 shows the nutritional composition of unmodified cow's milk differs in protein composition, fat composition, and carbohydrate and mineral concentrations to that of mature breast milk (Knipschidt, 1988).

Several authors, including the Australian College of Paediatricians, now recommend that infants not be introduced to cows milk prior to six months of age as a safeguard against nutritionally inadequate food (Stanway and Stanway, 1983, Knipschidt, 1986, Retallack *et al.*, 1994). Furthermore, the high protein and mineral content in cows milk puts a high load on the infants developing kidneys, the capacity of which is very limited (i.e. this becomes particularly critical when the loss of water is increased by fever or diarrhoea). (Knipschidt, 1986).

Due to both physiological (lack of salivary amylase) and developmental (e.g. the tongue thrust reflex reasons), and infant less than 6 months old should not be introduced to solids (Retallack *et al.* 1994).

Table 2.4 Nutritional composition of mature breast milk and unmodified cow's milk

Nutrient	Quantity	Average matured breast milk per 100 ml	Average cow's milk per 100 ml
Total protein	g	1.5	3.3
Protein composition			
Whey proteins	%	70	20
Casein	%	30	80
Fat	g	3.3	3.5
Fat composition			
Unsaturated fats	%	52	43
Saturated fats	%	48	57
Carbohydrates	g	7.0	4.7
Minerals	g	0.21	0.72
Energy	kcal	72	66
Protein calories	%	6	21

Other benefits to the infant by exclusive breast feeding include; fewer infections (i.e. helps protect against persistent and recurring gastrointestinal illnesses), fewer allergic diseases (i.e. by coating the lining of the gut with IgA, this not only fights infection but also prevents large protein molecules from leaking through the gut and into the

bloodstream, less coeliac disease, ulcerative colitis, fewer sudden infant deaths, less obesity, heart disease, dental decay, better jaw and mouth movements etc. (Stanway and Stanway, 1983). While benefits of breast feeding to the mother include; satisfaction, enjoyment, fulfilment, getting figure back, convenience, cost, birth control and the reduced risk of breast cancer (Stanway and Stanway, 1983).

Despite the strong supporting evidence for breast milk as the choice food for developing infants, not all mothers are eager or medically capable of breast feeding (Stanway 1983). While breast feeding by its nature is a natural process, Martin (1978) illustrated that some mothers experience problems such as; getting the baby to take the breast, painful engorgement of the breasts, painful stitches, insufficient milk and sore nipples during the first week. The author went on to mention that half of the mothers taking part in the study stopped breast feeding six weeks after the birth, almost a third in the first two weeks and only a quarter were still breast feeding at four months.

Since not all mothers choose to breast feed, a substitute feed in the form of infant milk formula is required (Retallack *et al.*, 1994). Today, highly developed powders are available and considered by many to be invaluable (manufacturers of baby food powders emphasise that breast milk is the best food for the baby). Since the middle of the twentieth century emphasis has been placed on the advice from paediatricians that baby food powder should approximate to the composition of breast milk (Stanway and Stanway 1983). Indeed, medical authorities advise against feeding infants below four months of age with unmodified cows milk for reasons mentioned earlier, and this has led to the development of so called "humanised" baby food powder, suitable for babies from the time that they are born (Retallack *et al.*, 1994). In practice, humanised baby foods are produced to match the average analysis of breast milk produced some fourteen days after partuition. (Knipschidt, 1986).

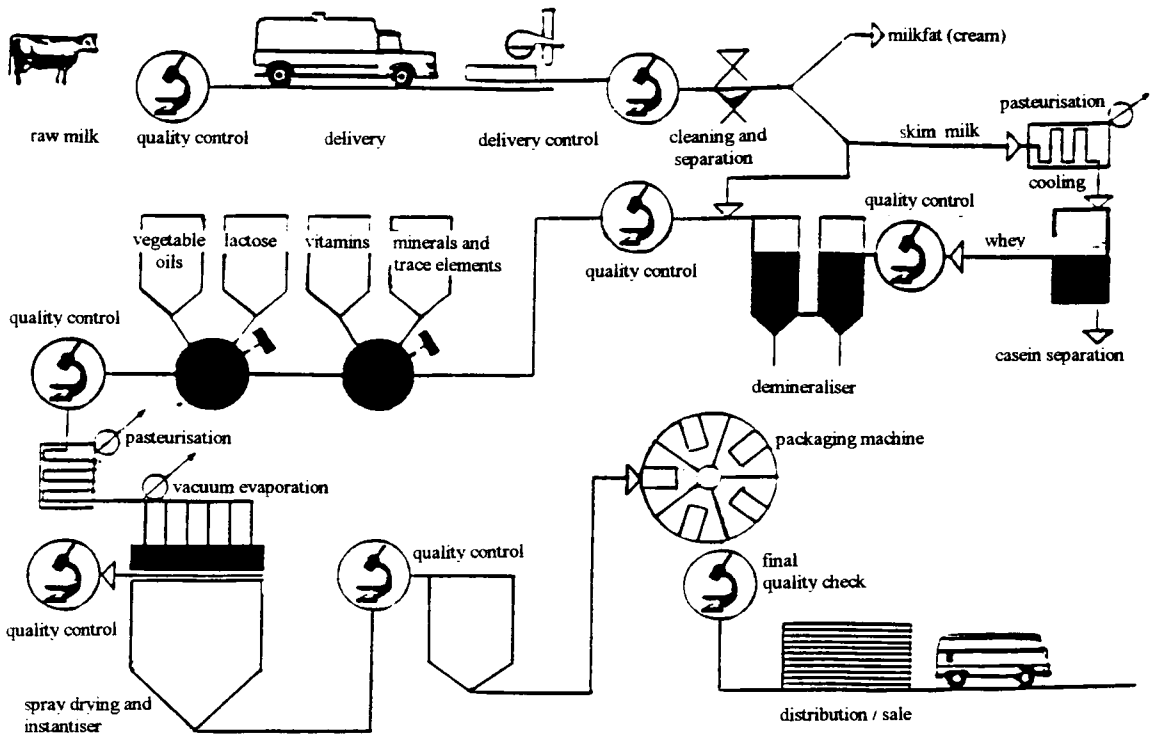
The production of IMF is steadily rising, and the milk powder industry in the UK produced some 343,000 tonnes of powder in 1987 amounting to some 11% of the total output of EEC and 6% of the world output (Mettler 1989). While Filer (1993) revealed that the US annual production of formula products for sale had escalated to 118,000 tonnes with greater complexity of ingredients. While the total solids content

of breast milk and cows milk is about the same there is considerable difference in composition (Table 2.4). It is therefore necessary to modify cows milk to obtain a product similar in analysis to breast milk (the shortcoming of manufactured baby food is that it does not possess immunological properties).

Great care must be taken in the manufacture of infant foods in order to safeguard the bacteriological quality of the finished food product. Figure 2.2 illustrates the infant powder manufacturing process. Raw milk destined for IMF production should be antibiotic free and should not contain $>10^4$ organisms ml^{-1} (Knipschidt, 1986, Von Wies,e 1992). As dry mixing of ingredients into the spray dried powder may lead to bacteriological contamination the modifications to the bovine milk should be incorporated during the spray drying process (Knipschidt, 1986).

After the necessary microbiological and chemical quality control checks, skim milk is produced through a process of standardising and clarifying. The composition of the skim milk is then adjusted by adding lactose and/or maltodextrin, whey powder (to offset the casein level), fat soluble vitamins A & D, iron, and vegetable oil (i.e. to balance the saturated to unsaturated fatty acids). The mix is then homogenised, pasteurised at 110°C with one minute holding and evaporated to 47/48% total solids (this high solid ratio to water facilitates spray drying). The temperature of the first effect in the evaporating chamber is 70°C (Knipschidt, 1986). The heat sensitive vitamin (C) is then added to the concentrate.

It is necessary to overdose in vitamin C as a portion is destroyed during drying. The concentrate should have a temperature of 60-70°C and should be spray dried using an air inlet temperature of 180°C. Although the air inlet temperature may be 180°C the temperature of the spray dried particles do not exceed the dew point, and hence there is no damage to the proteins. The powder is collected at the bottom of the fluidised bed, where the powder has been dried to less than 4% moisture or less than 0.5 a_w. The infant milk powder is then mechanically packaged prior to a final quality control check (Knipschidt, 1986, Mettler, 1989).



(Figure 2.2 adopted from Von Wiese 1992)

Figure 2.2 Stages involved in the manufacture of infant milk powder.

Despite the bactericidal temperatures employed during the spray drying process (particularly during the pasteurisation, evaporation and spray drying stages), several authors have demonstrated the ability of both spore forming (e.g. *Bacillus spp.*) and non-spore forming bacteria to survive these high temperatures (Naguib *et al.*, 1972, Thompson *et al.*, 1978, Mettler, 1989, Fleet, 1991).

Indeed, *Listeria monocytogenes*, *Salmonella spp.* and *Staphylococcus aureus* have been shown to be able to tolerate the spray drying process (Int'd Veld *et al.* 1994, Fleet 1991, El Dairoity 1989). Investigations by Crossley and Maltich (1949), showed that the microflora of spray dried milk is influenced by the initial population in the

milk, thermal treatment before and during spray drying , sanitation of equipment and post process contamination.

While, Mettler (1989) stated that all but bacterial spores should be killed by effective in process pasteurisation, such as HTST pasteurisation, and that the subsequent pathogenic presence of microorganisms in milk based powders is due to contamination after this pasteurisation process. However, this is not a plausable assumption as most *Bacillus* spp. are known to tolerate temperatures well in excess of this HTST pasteurisation process (section 2.2.4). The author described how certain critical control points in the spray drying process may fall foul to microbial contamination. The skills required in the unfailing production of pathogen free milk powders are collectively described as " Good Manufacturing Practice" and failures in this area led to a number of incidents of *Salmonella* food poisoning (see section 2.3.2). The present day drying techniques favours lower outlet temperatures which will favour a lower pathogen kill.

Mettler (1989) stated that the spray drying process itself cannot be relied upon to eliminate pathogens, as the bacteriological effect will depend on; (1) pathogen genus, species and/or serotype. (2) pathogen growth conditions, (3) product formulation, (4) feed concentrate total solids, (5) spray dryer inlet temperature and (6) spray drier outlet temperature.

2.3.2 The microbiological quality of infant milk formulae.

It has been conclusively shown that both milk and its by products (e.g. reconstituted infant milk formulae) are ideal growth media for microorganisms, as they are high in moisture, of nearly neutral pH and rich in essential nutrients (Mettler, 1989). It is for this reason that the hygienic quality of reconstituted and dried milk products is of paramount importance. Indeed, due to the low water activity level of dried infant milk powder (Piscky, 1992), indigenous microorganisms (e.g. spoilage and opportunistic pathogens alike) are not capable of regeneration in the dried powder (Mettler 1989).

Nevertheless, reconstituted baby foods are considered to be a food class of high risk due to the high susceptibility of the consumer population to enteric microbial pathogens, severe response to toxins and increased mortality (ICMSF 1986). However, there is no requirement that these foods should be sterile (ICMSF 1986).

A working party of the Scottish Food Co-ordinating Committee reported that in a preliminary survey of bacterial quality of dried milk in Scotland in 1987, counts of 12 types of baby formulae ranged from 0 to $4.5x10^6$ CFU ml^{-1} (Scottish Food Co-ordinating Committee, 1987). Dried milk products are known to be frequently contaminated with *Bacillus spp*. Becker and Terplan (1992) reported that when 261 samples of infant food distributed in 17 countries were examined for *B. cereus*, 54% of then were contaminated with *B. cereus* reaching levels of 0.3 to 600 g^{-1}. When samples contaminated with approximately 100 *B. cereus* cells ml^{-1} were reconstituted and incubated at room temperature (27°C), levels of 10^5 organism ml^{-1} were reached in 7-9 hours.

In 1980, Veda *et al.*. analysed 78 dried commercial baby foods in Japan and found that they contained $>10^2$ aerobic spore forming bacilli. The organisms isolated most frequently were *B. licheniformis* and *B. subtilis;* however, other species isolated included *B. cereus. B. pumilus, B. megaterium, B. circulans,* and *B. coagulans.* Shinagawa (1992) also reported a high incidence of *B. cereus* (42%) in powdered milk samples with counts ranging from 10^1 to 10^3 organisms g^{-1}. This author also reported that *B. cereus* enterotoxin was produced in milk samples within 12 - 18h when incubated at 20°C.

Concern about contamination with Gram negative bacteria introduced during reconstitution of these feeds means that many hospitals are now pasteurising infant feeds (Burnett *et al.* 1989). However, the counts of aerobic spore forming bacilli in feeds are little affected by pasteurisation and Burnett *et al.*. (1989) reported counts of 10^2 ml^{-1} post heat treatment.

Lovell (1981), Stadouders *et al.* (1982), Kwee *et al.* (1986) and earlier work by Crosseley and Johnson (1949), established that the bacterial flora of powdered milk consisted primarily of aerobic spore formers, thermoduric micrococci, thermoduric streptococci and sorynebacteria. This is quite relevant as *Listeria monocytogenes* was

at one point in time, classified as belonging to the corynebacteria. Two major studies of the microbiological quality of dried milk mix and milk substitute infant formula, one study in Canada by the Food and Drug Directorate (Collins-Thompson *et al.*, 1980) and another in the US by the United States Department of Agriculture(FDA 1967) have shown a geometric mean aerobic plate count of 25/ g^{-1} (dried milk mix) and 52 /g^{-1} (infant milk substitute).

A particular point of concern is the international "non-conformity" for acceptable microbial standards on the quality of infant formulae (IMF) (Table 2.5). Indeed, the codes (e.g. the Codes Alimentarius Commission of the Food and Agriculture Organisation of the UN, the World Health Organisation, the International Commission for Microbiological Specifications for Foods etc.) are advisory in nature and leave to the individual governments to decide what use they wish to make of them (Von Wiese, 1992).

Despite the normal bacteriocidal temperatures employed in the manufacture of IMF products and the on going total procedural commitments to product quality and safety, there have been a number of food related illnesses where infant milk powder (and not the method of reconstitution) have been implicated as the vehicle of infection (Table 2.6)

Both Collins (1986) and Rowe (1987) reported that the source of the contamination lay in a faulty spray drier. The levels of *Salmonella* in dried milks which were implicated in foodborne outbreaks were recovered in quite low numbers, usually in the range of 1 to 10 cells per 100 g of powder. For example, a count of 9 *S. newport* per 100 g were detected in the contaminated powder during the Canadian outbreak in 1986 (Mettler 1989). In the outbreak concerning *S. ealing* in 1987, a bacterial count of 3 cells per kg powder were found (Rowe, 1987).

After 3 reported cases of infection with *Salmonella* serotype t*enessee* in infants, the FDA ordered the recall of all powdered products from the factories on 4 November 1992. Gericke (1993) reported on an epidemic spreading of a multi-resistant *Citrobacter freundii* strain at a neonatal intensive care unit. One premature baby died of septic shock and the source of infection was traced to the infant formulae used on the ward. Kramer and Gilbert (1989) are the first and only authors to mention a

foodborne illness outbreak where a member of the *Bacillus spp.* (i.e. *B. subtilis*) was the aetiological agent concerned and infant milk formulae was the vehicle of transmission.

Table 2.5 National and international standards for the bacteriological quality of infant milk powder.

ORIGIN	COMMITMENT	AUTHORITY	TOTAL AEROBIC MESOPHILIC COUNT CFU/ g (30°C)	BACILLUS CEREUS
International	Recommended	FAO/WHO Codex	$m=10^3$ $M=10^4$/g	-
International	Draft	Alinorm 79/13	$m=10^3$ $M=10^4$/ g	-
International	Recommended	ICMSF	$m=10^4$ $M=10^6$/ g	$m=10^2$ $M=10^4$/g
European Comm.	New proposal	IDACE	$m=10^3$ $M=10^5$/g n=5 c=2	$m=10^2$ $M=10^3$/g
Germany	Standard	Diet Regulation	$\leq 5x10^1$/g	-
Switzerland	Standard	Regulation	$\leq 10^5$/g	$\leq 10^3$/g
Hungary	Agreement	Agreement	$\leq 10^4$/g	$\leq 10^2$/g
Netherlands	Standard	Regulation	$\leq 10^4$/g	$\leq 10^2$/g
Poland	Agreement	Agreement	$\leq 10^4$/g	$\leq 10^2$/g
Algeria	Agreement	Agreement	$m=10^3$ $M=10^4$/g	-
France	Standard	Decree	$\leq 5x10^4$/g	-
Spain	Standard	Regulation	$\leq 5x10^4$/g	-
Italy	Standard	Decree	$\leq 10^4$/g	-
Bulgaria	Agreement	Agreement	$\leq 10^4$/g	-
Romania	Agreement	Agreement	$\leq 10^4$/g	-
Finland	Standard	Regulation	$m=10^4$ $M=5x10^4$/g	$m=10^2$ $M=10^3$/g
Iran	Agreement	Agreement	$\leq 10^4$/g	absent/g^{-2}
Saudi Arabia	Standard	Regulation	10^4/g	-
Norway	Standard	Regulation	$m=10^3$ /g $M=10^4$/g	$m=10^2$/g $M=10^3$/g
Denmark	Standard	Regulation	$\leq 10^4$/g	absent/g^{-2}
CIS (formerly USSR)	SAN-PIN standard	Regulation	$\leq 2x10^2$/g to 10^4/g	$\leq 10^2$/g
CIS (formerly USSR)	COMECON standard	Regulation	$m=10^4$/g $M=5x10^4$/g	$m=10^2$/g $M=10^3$/g
Syria	Specification	Agreement	$\leq 10^5$/g	absent/25g
Portugal	Standard	Regulation	$\leq 10^4$/g	10^2/g
Isreal	Recommended	Code of practice	$\leq 10^4$/g	absent/30g
Australia	Standard	Regulation	$m=10^3$/g $M=10^4$/g n=5 c=2	$m=10^2$/g $M=10^3$/g n=5 c=1
Canada	Draft	Guideline	$m=10^3$ $M=10^4$/g n=5 c=1	$m=10^2$ $m=10^3$/g n=5 c=1

n=number of sample units taken for product control
c=number of sample units the bacterial count of which may be between m and M, the sample still being considered acceptable if the bacterial count of the other sample units m or less
m= threshold value for the number of bacteria: the result is considered satisfactory if the number of bacteria in all sample units does not exceed m
M= maximum value for the number of bacteria : the result is considered unsatisfactory if the number in one or more sample units is M or more

Given the susceptibility of the host population to enteric pathogens and the millions of tonnes of milk based powder produced annually world-wide, the record of the milk powder industry as a whole is a good one. However, few but the foolhardy would rule out the potential for pathogenic development (where present) in mistreated IMF products. Indeed, the potential underestimation of heat stressed microorganisms and ungerminated spores in milk powders, the non-conformity of the international standards for the microbial quality of infant formula, and the occasional finding of opportunist pathogenic bacteria in these products should serve as a warning to what may happen if standards are further relaxed (Read, 1982).

Table 2.6 Foodborne illness outbreaks where infant milk powder was implicated as the source of infection

Year	Country	Organism	Number affected	Reference
1968	USA	*Salmonella newbrunswick*	12	Collins *et al.*, 1968
1972	?	*Pseudomonas aeruginosa*	2	Read 1982
1976	?	*Staphylococcus enterotoxin A*	?	Read 1982
1977	Austria	*Salmonella bredeney*	?	Mettler 1989
1977	Trinidad	*Salmonella spp.*	?	Kramer and Gilbert 1989
1985	UK	*Salmonella ealing*	62	Rowe 1987
1986	Austria	*Salmonella isangi*	21	Mettler 1989
1988	Austria	*Salmonella isangi*	13	Mettler 1989
1988	France/UK	*Salmonella bredeney*	?	Mettler 1989
1989	UK	*Bacillus subtilis*	1	Kramer and Gilbert 1989
1993	Candada	*Salmonella tenessee*	1	Louie 1993
1993	Germany	*Citrobacter freundii*	1	Geriche and Thurn 1994

2.3.3 Potential health problems associated with the improper reconstitution of infant milk formulae.

The improper reconstitution of baby powders with microbiologically poor quality water and /or with contaminated feeding bottles is a global problem, but particularly in developing countries (Motarjemi, 1993, Long *et al.*, 1994). World-wide (excluding China) it is estimated that 1400 million episodes of diarrhoea occur annually in children under the age of 5 years (Motarjemi, 1993). In 1990, over 3 million of such children died as a result (WHO/hst/1992). Indeed, up to 70% of diarrhoegenic episodes could be due to pathogens transmitted in food (Esrey, 1989). Various pathogens have been identified as causing foodborne diarrhoea diseases including; *Escherichia coli, Shigella spp., Salmonella spp., Vibrio cholera, Campylobacter jejuni*, protozoa such as *Entamoeba histolytica, Cryptosporidium* and also enteric viruses. In addition, *B. cereus, S. aureus , C. perfringens* and helminths are common foodborne pathogens that cause disease frequently accompanied by diarrhoea (Motarjemi, 1993).

Infant formula contamination is one of the major contributors to diarrhoeal diseases and the malnutrition associated with it. Moreover, this form of foodborne illness can cause severe and/or long lasting damage to health including acute, as well as chronic diseases affecting the renal, articular, cardiovascular, respiratory, and immune system (Motarjemi, 1993). Indeed, a poor intake, aggrevated by loss of nutrients from vomiting, diarrhoea, malabsorption and fever over an extended period (persistent diarrhoea), leads to nutritional deficiencies with serious consequences for the growth and immune system of the infants and children. Thus, an infant whose resistance is suppressed becomes vulnerable to other diseases (including respiratory infections) and is subsequently caught in a circle of malnutrition and infection (Motarjemi, 1993).

Whole generations of bottle fed babies have recently grown to adulthood in affluent countries without experiencing the epidemic marasmus (i.e. wasting away of the body or failure to thrive due to malnutrition) and kwashiorkor (i.e. a nutritional disorder of infants where the diet is persistently deficient in essential protein) which appears to be the fate of many bottle fed infants in the poorer societies of the developing world

(Surjono *et al.*, 1980). The authors went on to say that the culprit was not the milk, per se but the process of bottle feeding.

The use of milk based infant formulae became common the late 19th century with the increase employment of women. However, in the US in 1905, about 1/5 of deaths of all ages from all causes were of infants under 1 year of age and of these infant deaths, 40% were from diarrhoea and enteritis diseases thought to be food borne in origin. Moreover, bacteriological examination of milk made at the Hygiene Laboratory of the Public Health and Marine Hospital Service during the summer of 1906 showed average plate count of $2.2x10^8$ organisms ml^{-1} compared with today's maximum limit of $2.0x10^5$ CFU ml^{-1} (Rosenau *et al.*, 1909).

Many epidemiological studies has shown that breast fed infants have a significantly lower incidence of gastro-enteritis than infants that are bottle fed. Sazawal *et al.* (1992) demonstrated that breast fed babies in North India are 16.5 times better off (if breastfed) compared to bottle fed babies, as regards contracting and/or developing persistent diarrhoea. This finding of breast feeding having an influential effect on preventing diarrhoea associated illnesses and/or deaths has been supported by a number of authors (Gunn *et al.*, 1979, Surjono *et al.*, 1980, Bulletin WHO 1989, de Zoysa, 1991, Long *et al.*, 1994).

Breast feeding compensates for the loss of water and nutrients that occurs during diarrhoea (Moterjemi 1993). Further work by Edwards *et al.* (1994) demonstrated that the difference in faecal flora (i.e. breast fed babies are more likely to have bifidobacterium and lactobacilli as the predominant micro-organisms where bottle fed infants tend to have more enterobacteria, streptococci and bacteriodes *spp.*) maybe another reason for the increased incidence of enteropathogenic organisms and infectious diarrhoea in formula fed babies.

Strategies proposed for the prevention of diarrhoeal diseases and associated malnutrition in developing countries include; breast feeding or improving water supply and sanitation, education of food handlers (particularly mothers) in food safety. In view of the protective effects of breast milk against diarrhoea and malnutrition, reports by de Zoysa, (1991) and the WHO (Bulletin of the WHO 1989) recommends the continuation of breast feeding for at least 2 years.

Apart from the microbiological impact of improper and unhygienic reconstitution of infant formulae, serious dilution errors may arise in the rehydration of this infant power. Grant (1994) reported of a 4 month old infant demonstrating signs of hyponatraemia, which was caused by water intoxication secondary to inappropriate milk formulation. While research by Lucas (1992) illustrated that infant formula fed babies followed a dissimilar physiological pattern of growth to that of breast fed babies, where the former group of infants had a significant increased body weight and skinfold thickness gains. Furthermore, research by Masaiger (1993) in the Republic of Yemen, revealed that many mothers try to dilute the powdered milk to make it suitable for the child, this practice may affect the nutritional status of the infant as over dilution reduces many essential nutrients.

The promotion and advertisement of IMF must be in accordance with the WHO's International code of marketing of breast milk and in the UK by the statutory instruments Act 1995 (Statutory Instruments No.77, 1995). While these codes particularly emphasise the importance of the requirement for the word "Important Note" where a statement concerning the superiority of breast feeding and a statement recommending that the product be used only on advice of an independent person qualified in medicine, nutrition or pharmacy or having professional qualifications in maternal or child care studies, studies by many researchers reveal that improper promotion and advertisement of baby food products occurs in developing countries, including the Republic of Yemen, Pakistan, Thailand, and the Dominican Republic (Brown, 1989, Misaiger, 1993).

Indeed, the majority of paediatricians (70%) in the Republic of Yemen agree that the best method to promote breast feeding is to restrict the marketing of formula to one or two brands, while more than 57% believed that IMF should only be given by prescription.

3 Materials and Methods

3.1 Microorganisms

3.1.1 Sources of cultures

The following bacterial species were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale, London: *Listeria monocytogenes* serotype 4b (NCTC 11994), *Listeria monocytogenes* serotype 4b (NCTC 10357), *Listeria monocytogenes* serotype 1a (NCTC 10357), *Listeria seeligeri* (NCTC 11856), *Listeria murrayi* (NCTC 10812), *Listeria grayii* (NCTC 10815), *Listeria welshimeri* (NCTC 11851), *Listeria ivanovii* (NCTC 11846), *Listeria innocua* (NCTC 11289), *Rhodococcus equi* (NCTC 1612), *Staphylococcus aureus* (NCTC 6681), *Bacillus cereus* (NCTC 11143), *Bacillus cereus* (NCTC 11145), *Salmonella enteritidis* NCTC 8515, *Bacillus cereus var mycoides* (NCTC 926), *Bacillus subtilis* (NCTC 3610), *Bacillus pumilus* NCTC 10337), *Bacillus brevis* (NCTC 2611), *Bacillus laterosporus* (NCTC 7579), *Bacillus lentus* (NCTC 4824), *Bacillus sphaericus* (NCTC 7582), *Bacillus circulans* (NCTC 9432), *Bacillus licheniformis* (NCTC 10341) and *B. megaterium* (NCTC 6005). Other organisms employed during this study were two strains of *Bacillus amyloliquefaciens* (NCIMB 10785 and NCIMB 12077) which were obtained from the National Collection of Industrial and Marine Bacteria Ltd, Aberdeen, Scotland.

3.1.2 Stock cultures

A well isolated colony of each strain was transferred to Tryptone Soya Agar supplemented with 0.6% of Yeast Extract (TSYEA) slopes and incubated at 37°C for 24 hours. After this incubation period, the slopes were subsequently stored at 4°C and/or freeze-dried in a pre-sterilised ampoule which contained Horse Serum supplemented with 6.9% Glucose and 23.3% Nutrient Broth (section 3.5.3). These slopes and/or freeze dried cells were employed as culture stocks. The stock cultures

were periodically checked for purity by subculturing onto solid agar media, the resulting surface cultures were examined for characteristic cell and colony morphology, and for the unique set of physiological/biochemical properties. Prior to each experiment, the stock cultures were activated twice by growing the test organisms on culture media. This was achieved by either aseptically transferring some sloped stock culture to the surface of a TSYEA plate via the streak plate technique (section 3.5.1), or resuscitating the freeze dried cells by resuspending these cells in sterile Nutrient Broth prior to streak plating onto TSYEA. The culture media were incubated at 37°C for 24 hours.

3.2 Materials and Apparatus

3.2.1 Food samples

The food samples used in this study composed of a selection of reconsitituted infant milk formulae (UHT and designated as *ready-to-feed*), infant milk powder (IMF) and UHT milks which had been purchased from a local retail outlet. The IMF were reconstituted at various water temperatures in sterile water (section 5) and analysed without a subsequent sterilisation treatment (i.e. the powdered samples were rehydrated according to the instructions laid down by the manufactures. Depending on the type of investigation being performed these reconstituted infant milk formulae were either tyndallised to sterility (i.e. three successive days steaming at 100°C for 30 mins) or simply reconstituted at different sterile water temperatures in pre-sterilised infant feeding bottles or Duran bottles. The UHT rehydrated infant milk formulae and UHT milks were tyndallised to sterility. Prior to opening of each milk product, vinyl gloves wiped with 70% ethanol were worn. The sample package was wiped with ethanol to avoid contamination. To ensure homogeneous distribution of the infant milk powder on reconstitution, sterile glass beads were placed in the food containers and the samples were shaken 25 times for 15 seconds through an excursion of 30 cm.

The package integrity of each food sample was checked for cracks and/or leaks prior to bacteriological analysis.

The IMF which had been prepared at the Royal Hospital for Sick Children (Yorkhill, Glasgow) and/or at Ruchill Hospital (Ruchill, Glasgow) were transported to the laboratory in a cooler bag containing two ice packs and a maximum/minimum thermometer which had been included to regulate and record the storage temperature (at -1°C ± 2°C) in transit respectively.

3.2.2 Pipettes

Two different automatic pipetter models were used throughout this study namely the Finnpipette Digital (used for the dispensing of aliquots greater than 0.1 ml) and the Eppendorf (an adjustable pipetter which dispensed sample volumes of 10 to 100 µl). These instruments dispense an adjusted volume of liquid which is registered on a read-out window. They operate on an air displacement principle (air interface) and use detachable, disposable tips. The pipetters were cleaned at regular intervals by swabbing of the barrel secton with 70% ethanol and their sterility was challenged during each experiment by dispensing sterile water onto culture media. The pipette tips were autoclaved to sterility by steaming under pressure at 121°C for 15 mins. Other pipettes employed include the pre-sterilised Pastettes (a transparent plastic non automatic pipette graduated at intervals along the length of the outside wall). After use, the pipette tips (and contaminated pastettes) were placed in polythene pipette jars containing disinfectant solution (hypochlorite solution containing 2500 ppm chlorine).

3.2.3 Spreaders

Disposable, pre-sterilised plastic L-shaped spreaders (D517- a product of the LABM Company) were used throughout the study. Decontamination of the plastic spreaders was achieved by placing the soiled spreaders in an autoclavable plastic bag and then sterilising. A sterility check was performed on randomly selected sterile spreaders

during each experiment by spreading 0.1 ml aliquots of sterile water across the surface of a non selective plating medium (TSYEA).

3.2.4 Disposable universal containers

For convenience and to ensure sterility, disposable universal containers (Cat No:275/0472/01, a product of BDH) were employed where possible. By design, they are sterile plastic screw capped bottles with a capacity to hold approximately 20 ml of sample. After use the contaminated bottles were deposited in an autoclavable bag and then sterilised.

3.2.5 Glassware

Cultivation of microbial cells occurred in ether 250ml or 500 ml Erlenmeyer conical flasks which were obtained from the University Chemistry store. Non-absorbent cotton wool was positioned in the mouth of each flask and a layer of aluminium foil wrapped over this seal. Other glassware employed during this study include: 28 ml McCartney bottles (which were subsequently used for thermal inactivation studies) and 7 ml Bijou bottles (which were generally used for performing serial dilutions), and 250ml / 500 ml Duran Bottles (which were used for the preparation and sterilisation of culture media and for the reconstitution of infant milk powders). All glassware were sterilised by autoclaving at 121°C for 15 mins.

3.2.6 Infant feeding bottles

The infant feeding bottles were purchased from local retail outlets and consisted of transparent plastic bottles (with the capacity of hold approximately 280 ml of liquid), silicone teats, plastic sealing discs, plastic locking rings and plastic dome cap. Before use, the teat and other components were thoroughly washed and brushed in warm soapy water. The teat was rubbed inside and out with salt and the bottle components

were then sterilised by placement in either the infant feedtime steam steriliser (Figure 3.1), the microwave feeding bottle steam steriliser (Figure 3.2) or the complete feedtime steam steriliser (Figure 3.3), as described in section 3.2.7. The first two sterilising methods sterilise the infant feeding bottles through the application of heat while the latter method uses a chemical sterilisation approach. All infant feeding bottle components were checked for any structural defect prior to use.

3.2.7 Infant feeding bottle sterilisation equipment

The Boots® Feedtime Steam Steriliser is an automated heating system and holds up to 6 feeding bottles, standard or wide necked, and accessories (Figure 3.1). Contaminated feeding bottles were sterilised by steaming (100°C) over a 9 minute exposure time period (section 5.4).

Figure 3.1 Boots® Feedtime Steam Steriliser

Figure 3.2 Boots® Microwave Feeding Bottle Steam Steriliser.

Figure 3.3 Boots® Complete Baby Feedtime Steriliser.

The Boots® Microwave Feeding Bottle Steam Steriliser consists of a sealed plastic compartment capable of holding 4 feeding bottles and accessories (Figure 3.2). The used feeding bottles were emptied and placed in this microwavable plastic container (which contains water), the compartment was positioned inside the microwave oven (Toshiba ER-686.E/EW) where it was subsequently heated at full power for 8 minutes. Steam is produced and retained in the container by the heating effect of the microwaves and the microbial cell flora present in any IMF biofilm are heat inactivated.

The Boots® Complete Baby Feedtime steriliser is a chemical treatment process (Figure 3.3). Infant bottles are sterilised by being submerged in a disinfection solution (normally a sodium hypochlorite solution containing-125 ppm cholorine and otherwise known as Milton solution). It consists of a strong clear plastic tank which can hold up to 6 contaminated infant bottles and accessories. A hygienic plastic tray holds the teats in position, thus enabling their removal from the sterilising solution without either contaminating the sterilising solution or the sterilised teats. All three infant bottle sterilisers are products of Boots® the Chemists, UK. Ltd.

3.2.8 pH meter

For measuring the pH, a pH meter (7010, Kent-Electronic Instruments Limited) was employed as outlined in the manufacturers instructions.

3.2.9 Spiral plater

The Spiral Plater model B (Spiral Systems Inc. ®) was used by following the instructions laid down by the manufacturer. It is an automated diluting and dispensing system, which repetitively and predictably distributes 35 µl (for a 10-cm plate diameter) of the designated sample on the surface of a rotating agar plate from the centre to the edge in an ever decreasing amount. Every sample is deposited in the form of an Archimedes spiral in such a manner that the volume (µl) dispensed on any

portion of the plate is known and always the same. After a suitable incubation period, colonies appear on the lines of the spiral and the bacterial concentration (cfu/ml^{-1}) is determined by counting the colonies on a countable segment of the plate, and dividing this number by the volume of the sample contained in the areas enumerated.

The counting of colonies by the spiral plate method was carried out according to the instructions laid down in the Spiral Plater User Manual (Spiral Systems, Inc). In order to quantify the bacterial concentration (cfu/ ml^{-1}) present, any sector was chosen on the 10-cm Spiral System counting grid (Figure3.4) and the colonies were counted from the outer edge toward the centre until 20 colonies had been counted. The count was completed by counting the remainder of the colonies contained in the segment which the 20th colony was observed. This number was recorded along with the number of the segment that included the 20th colony marked on Figure as 3c, 3b , 3a, 4c, 4b, 4a, or total. In order to balance any irregularities in sample deposition, the same segments in the opposite sector were counted and recorded also. If there were not a total of 20 colonies in the 4 segments of the sector, then all of the colonies on the entire plate were counted and recorded with a T to indicate a total plate count.

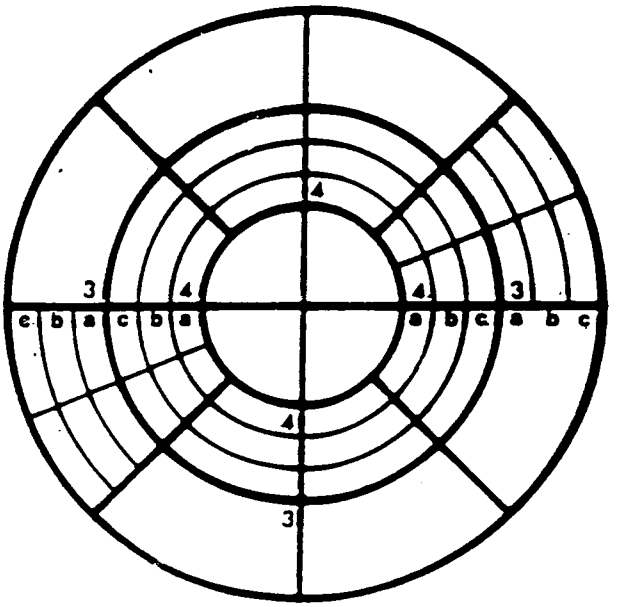


Figure 3.4 10-cm Spiral System counting grid.

The bacterial density was estimated by dividing the total count obtained by the volume contained in all the segments counted. If there were more then 75 colonies in the segments containing the 20th colony, the count was made by counting circumferentially adjacent annular segments in all sectors, starting with sector 1 until at least a total of 50 colonies were counted.

3.3 Diluents and Media

3.3.1 Diluent

The diluent used throughout this work was a Phosphate buffered Saline solution (PBS: 0.01 M sodium phosphate (pH 7.2), 0.15 M NaCl). The stock solution was prepared by making up 0.5 M $NaH_2Po_4.2H_2O$ (i.e. 39g in 500 ml H_2O) and 0.5 M Na_2HPo_4 (i.e. 35.49g in 500 ml H_2O) to 500 ml and adjusting the pH to 7.2 on the pH meter. A working solution of 0.01 M sodium phosphate (at pH 7.2) was prepared by adding 10 ml of stock solution to 990 ml of distilled water. The addition of 2.1g NaCl to 500 ml 0.01M sodium phosphate (pH 7.2) achieved the final PBS working solution or diluent.

3.3.2 Media

The bacteriological media employed during this research included: Tryptone Soya Broth supplemented with 0.6% Yeast Extract (TSYEB), Tryptone Soya Agar supplemented with 0.6% Yeast Extract (TSYEA), Nutrient Agar supplemented with 5 mg L^{-1} $MnSo_4.4H_20$ (NMA), *Listeria* Selective Agar (LSA-Oxford formulation), *Bacillus cereus* Selective Agar (BCSA), Blood Agar to which was added either defibrinated horse or sheep blood (BA), *Listeria* Selective Enrichment Broth (LSEB - Food and Drug Administration Approved Formulation/primary selective stage), *Listeria* Enrichment Fraser broth (LEFB-Food and Drug Administration Approved Formulation/secondary selective stage), University of Vermont Media (UVM1 and

UVM11, a United States Department of Agriculture Approved-primary and secondary selective enrichment broth respectively), Brain Heart Infusion Broth (BHI), Nutrient Broth No.1 and No. 2.

All culture media were purchased from Oxoid (Unipath Ltd., Basingstoke, England) and were prepared according to directions laid down by the manufacturer. The media was steamed and autoclaved at 121°C for 15mins, prior to cooling and holding in a preheated waterbath at 45°C. Approximately 15 ml of Agar medium was then aseptically poured into a 10 cm triple vented sterile Petri dish and allowed to solidify. Plates were then stored in an inverted position in a 37°C incubator to facilitate drying of any residual surface condensate. Other specialised media included in the study were Starch Agar, Casein Agar and Basal Synthetic Medium and they were prepared as follows:

3.3.2.1 Starch agar

A 10% solution of soluble starch was prepared in water and steamed for 1 hour. This solution (20 ml) was added to 100 ml of melted (sterilised) Nutrient Agar (45°C) and poured into the Petri dishes. The plates were stored as mentioned above.

3.3.2.2 Casein agar

Skim milk powder was reconstituted according to the manufacturers instructions and tyndallised (section 3.13). An equal volume of this tyndallised reconstituted skim milk (200 ml) was aseptically added to sterilised double strength Nutrient Agar (which had been equilibrated at 55°C). The mixture was then let cool to 45°C prior to aseptically pouring approximately 15 ml into the triple vented Petri dishes. The plates were stored as mentioned above.

3.3.2.3 Basal medium

This was used when investigations were being made into the ability of bacteria to utilise various carbon (i.e. lactose, glucose and/or maltodextrin) sources. The Basal Medium was prepared by adding; Ammonium dihydrogen orthophosphate (1.0g), Potassium chloride (0.2g), Magnesium sulphate (0.2g) and Yeast Extract (0.2g) into 1 Litre of distilled water. A 1.6% Bromocresol purple (6ml) was then added and the pH was adjusted to 7.2 using 2N NaOH. This Basal Medium was then transferred into the relevant vessels and sterilised by autoclaving at 121°C for 15 mins. The test carbohydrate was filter sterilised and then aseptically added in the desired volume to the sterilised Basal Medium. To achieve a concentration of 3.8% Maltodextrin Basal medium; add 10g Maltodextrin to 100 ml of sterile distilled water (heat gently to 50°C to dissolve) and filter sterilise, then aseptically transfer 38 ml of this 10% Maltodextrin solution to 62 ml of sterilised Basal medium (i.e. 3.8% Maltodextrin Solution).

3.4 Materials

3.4.1 Maltodextrin

Maltodextrins are added to a variety of follow on infant milk formulations (especially in infant products formulated for premature babies, or infants with milk intolerances and/or gastro-intestinal disturbances). However, it has been documented that starch, either naturally or artificially present in a food product may stimulate diarrhoeal enterotoxin production by indigenous *Bacillus cereus* (Garcia-Arribas and Kramer 1991). As maltodextin is a hydrolysed derivative of starch, a study was underataken to estabilish whether or not maltodextrin content correlated with diarrhoeal enterotoxin production in a variety of products. Maltodextrin (C# PUR 01915) supplied by Cerstar UK Ltd, is a fully soluble white powder and is derived from the spray drying of a purified aqueous solution of nutritive carbohydrates, obtained by the

enzyme hydrolysis of starch. It is used in the formulation of baby foods and was subsequently employed in this study to investigate diarrhoeal toxin production by strains of *Bacillus cereus*. The technical information supplied with the maltodextrin showed that it has a carbohydrate composition (% sugar base) of dextrose (1%), maltose (5%), maltotriose (8.5%) and higher sugars (85.5%).

3.5 Methods

3.5.1 Conventional plate count methods

The conventional spread, pour and streak plate techniques were performed according to Collins *et al.* 1989. The counting of colonies was executed according to the guidelines recommended by Collins *et al.* 1989.

3.5.2 Preparation of the samples

To prepare the food samples, 25 g of infant milk formula was added to either a pre-sterilised infant feeding bottle or a 250 ml Duran bottle (containing glass beads) each containing 250 ml of sterile distilled water (1:10 wt/vol). The reconstituted IMF were stored under the appropriate conditions and 10 ml aliquots were aseptically removed at the designated time intervals where they were then stored on ice (placing the sample in ice reduces the temperature of the sample to a level where it slows down the cells metabolism, thus fixing the cell concentration). Decimal serial dilutions of this sample were prepared by aseptically transferring the 1.0 ml volumes to sterile 9.0 ml aliquots of PBS. This mixture was vortexed into suspension prior to further serial transfers. Bacterial broth cultures were serially diluted in PBS as mentioned above.

3.5.3 Freeze drying of cultures

The bacterial cultures were freeze dried by the technical staff of Strathclyde University according to manufactures instructions.

3.5.4 Opening of ampoules

Opening of the ampoules was achieved by following the instructions laid down by the National Collection of Type Cultures. The Nutrient broth culture suspension was then streaked to single colonies on a TSYEA plate.

3.5.5 Biochemical test kits

These microbiological identification kits consists of rows of microtubes or disks filled with various freeze-dried substrates which are designed to either detect the presence of specific enzymes and/or fermentation of a sugar or alcohol. These microtubes were rehydrated with bacterial suspensions, and after a predetermined incubation period, the results were recorded as colour changes (which occurred due to either acid production during fermentation or following the addition of reagents). Some microtubes require the addition of sterile mineral oil. The result from each microtube were collated to form a unique series of numbers, this specific numerical sequence was interpreted using an identification table and/or computer program (e.g. APILAB plus).

3.5.5.1 MICRO-ID *Listeria*

The MICRO-ID *Listeria* test Kit (Organon Teknika Corporation, Durham, N.C.) is a system designed for the identification of *Listeria* species. The test kit consists of a plastic tray with 15 wells, each containing a filter-paper disk impregnated with reagents that can detect the presence of specific enzymes and/or metabolic products

produced by *Listeria* species. The MICRO-ID *Listeria* test kit was used according to the manufactures instructions.

3.5.5.2 API-CORYNE

The API-CORYNE kit (API BioMérieux, La Balme -les-Grottes,France) is a system which allows the identification of *Coryneform* bacteria in addition to *Listeria* species. It consists of a plastic strip with 20 microtubes containing dehydrated substrates for the demonstration of enzyme activity or the fermentation of sugars. The API-CORYNE kit was used according to the manufacturers instructions.

3.5.5.3 API-*LISTERIA*

The API-*LISTERIA* test kit (BioMérieux,La Balme-les-Grottes, France) is a system for the identification of *Listeria* species and consists of 10 microtubes containing dehydrated substrates which enable the performance of enzymatic tests or sugar fermentation's. An in-depth evaluation of API *LISTERIA* as a new and promising one day system for the identification of *Listeria* isolates has been performed by Bille *et al.* (1992).

The API-*LISTERIA* system consists of the following 10 tests: differentiation between *L. innocua* and *L. monocytogenes*, based on the presence of acrylamidase (DIM) test), hydrolysis of esculin, presence of α-mannosidase, the acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate and D-tagatose. The API-*LISTERIA* system was used according to the manufacturers instructions.

3.5.5.4 API 50 CHB and API 20 E

The API 50 CHB (*Bacillus*) kit (BioMérieux,La Balme-les-Grottes, France) is supplemented with the first 12 tests of the API 20 E (*Enterobacteriaceae*) for the

identification of *Bacillus species*. Both kits being set up after the organism had been confirmed as a member of the *Bacillus* genus by microscopic observation for endospores and sporangial morphology. The development of the system is described by Logan and Berkeley (1981).

The system is based upon the API 50 CHB kit which comprises the API 50 CH gallery, containing 49 dehydrated carbohydrates and one control in 5 strips of 10 cupules, and an ammonium salts inoculating medium (API 50 CHB medium) containing phenol red as indicator. The API 50 CHB and API 20 E galleries were used according to the manufacturers instructions.

Moreover, the API 50 CHB medium is a ready to use medium that enables the fermentation of the 49 carbohydrates on the API 50 CH strip to be studied. In principle, a suspension is made in the medium with the microorganism to be tested and each tube is inoculated. During incubation, carbohydrates are fermented to acids which produce a decrease in pH, detected by a colour change of the indicator. The results make up the biochemical profile of the strain and are used in its identification or typing.

3.5.5.5 Interpretation of the results from the API test kits using the APILAB Plus software package.

The results obtained from the API-CORYNE, API-*LISTERIA* and API 50 CHB (supplemented with API 20 E) test systems were interpreted by using the APILAB PLUS software (BioMerieux, France), which is a bacterial identification program.

According to the reference manual (API BioMerieux, France), the APILAB Plus software interprets the biochemical profiles obtained from the identification strips. Calculations are made according to Bayes theorem and include for each profile, the percentage of identification (% id) and the T index which compare the observed profile to taxa in the database.

The percentage of identification (% id) is an estimate of how closely the profile corresponds to the stated taxon relative to all the other taxa in the database. The T

index is an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon.

Based on the % id (or the sum of the % id) and the T index (or the average of the T indexes) the quality of identification is classified as follows:

Excellent identification % id $\geq$ 99.9 and T $\geq$ 0.75

Very good identification % id $\geq$ 99.0 and T $\geq$ 0.5

Good identification % id $\geq$ 99.0 and T $\geq$ 0.25

Acceptable identification % id $\geq$ 80.0 and T $\geq$ 0

The identification is: (a) to the species level if 2, 3 or 4 taxa belong to the same species have been selected; (b) to the genus level if 2, 3 or 4 taxa belonging to the same genus have been selected; low discrimination if 2, 3 or 4 taxa belonging to different genera have been selected; (d) not reliable if the sum of the % id is less than 80%; and (e) presumptive if the strain must be sent to a reference centre for supplementary identification. The profile is doubtful if a taxon having several tests against the identification is present among those selected. Finally the profile is unacceptable if it is not close to any taxa of the data base.

3.5.6 Haemolysis

A section from a well isolated colony of the test culture was inoculated by the stab technique into Horse Blood Agar. These stabbed plates were then incubated for 24 to 48 hours at 35°C in an inverted position. After this incubation period, the extent of haemolytic activity was characterised by: (a) γ-haemolysis, where no visible change occurs in the Blood Agar as a result of colony growth; (b) α-haemolysis, where a greenish zone is produced around the colony's outer margin and no zone of clearing is observed; (c) α' (Alpha prime) haemolysis, where the colonies are surrounded by an area of clearing (haemolysis) which resembles that of β-haemolytic activity but with a hazy outline and unaltered red blood cells within the haemolytic area; (d) β-

haemolysis, where the colonies are surrounded by a much larger, clear haemolysed zone in which all the red blood cells have been destroyed (Collins *et al.*, 1989).

3.5.7 CAMP test

The Christie-Atkins-Munch-Peterson test CAMP test (Lovett, 1988) was performed by streaking weakly β-haemolytic *Staphylococcus aureus* (NCTC 6681) and *Rhodococcus equi* (1612) vertically in one direction on a plate of Blood Agar containing 5% sheep's blood. Separate vertical streaks were made so that it was possible to streak the test organism horizontally between them, without quite touching them. After 24 and 48 hours incubation at 35 °C, the plates were examined for haemolysis in the zone of influence between the two streaks. *L. monocytogenes* haemolysis is enhanced in the vicinity of the *S. aureus* streak; *L. ivanovii* haemolysis is enhanced in the vicinity of the *R.equi* streak; and haemolysis of *L. seeligeri* is enhanced near the *S. aureus* streak. The other *Listeria* species were unaffected by the heamolytic activity of either of these cultures (i.e. *S.aureus* and/or *R. equi*). The CAMP test differentiates *L. ivanovii* from *L. seeligeri* and can differentiate a weakly haemolytic *L.seeligeri* (that may have been interpreted as nonhaemolytic) form *L. welshimeri*. Isolates giving reactions typical of *L. monocytogenes*, except for the haemolysin production, should be CAMP- tested before they are identified as nonhaemolytic *L. innocua*.

3.5.8 Catalase test

The catalase test was executed by emulsifying some of the 24 hour test culture in 0.5 ml Tween 80 in a 7 ml Bijou screw capped bottle. Afterward, 0.5 ml of 20 vol hydrogen peroxide was added and the cap was immediately replaced. Effervescence indicated the presence of catalase. The test must never be carried out on an uncovered slide as the effervescence creates aerosols. Cultures grown on low carbohydrate medium give the most reliable results.

3.5.9 Oxidase test (cytochrome oxidase test)

The oxidase test was performed by using commercial Oxidase Touch Sticks (an Oxoid product). In this method, one end of the stick is impregnated with a solution of N,N-dimethyl-ρ-phenylenediamine oxalate, ascorbic acid and α-napthol. The impregnated end of this stick was touched against a section of the colony under study. The stick was examined after 3 minutes. If the test organism produced cytochrome oxidase, the enzyme specifically combines with N,N-dimethyl-ρ-phenylenediamine oxalate and α-napthol and produces a blue-purple colour.

3.5.10 Tumbling motility

A well isolated colony was inoculated into 5 ml BHI broth and incubated at room temperature (approximately 25°C) for 24 hours. After incubation, a wet mount was prepared by placing a drop of the broth culture onto a glass slide and then covering with a cover slip. The wet mount was then examined with a phase contrast microscope which permits an observation of tumbling motility to be made. Tumbling motility or head-over-heels motility is a unique characteristic of *L. monocytogenes*. The temperature of incubation is critical to the successful outcome of tumbling motility.

3.5.11 Gram stain

A Gram stain reaction was performed according to the standard instructions described by Collins *et al.* 1989.

3.5.12 Spore Stain

The spore stain was carried out according to the instructions described by Parry *et al.* 1988.

3.5.13 Growth in 7.5% sodium chloride

Single isolated colonies of the test organism were inoculated into TSYEB containing 7.5% NaCl. After a 24 hour incubation period at 30°C, the culture was visually examined for turbidity (a turbid culture was indicative of growth).

3.5.14 Starch hydrolysis

A loop touched to a colony of the strain on TSYEA was streaked across a starch agar plate (3.3.2.1) which was then incubated at 30°C for 4 days. The plate was then flooded with a 1:5 dilution of Lugol's iodine solution. Unchanged starch turns blue-black; hydrolysis of starch was indicated by a clear zone around the streak. Weakly positive strains may not produce a visible zone around the colonies and, in the case of apparent negatives, some of the growth was scraped off to establish whether there was a clear zone underneath the culture growth.

3.5.15 Casein hydrolysis

A Casein Agar plate (3.3.2.2) was streaked with a loop previously touched to a colony of the strain under study. The plate was incubated at 37°C for 1 to 14 days. Clearing around the streak (or beneath the growth in the case of weakly positive strains) was looked for and indicated decomposition of the casein.

3.5.16 Growth in the presence of 0.001% lysozyme

A tube of 0.001% lysozyme in Nutrient Broth was inoculated with the strain being tested and incubated at 37°C. The tubes were examined at regular intervals up to 14 days for turbidity indicating the ability of the strain to grow in this medium.

3.5.17 Lecithovitellin/ lecithinase production

The test utilises the hydrolysis of the phospholipid lecithin of egg yolk (Colmer 1947) by the as yet imprecisely defined action of phospholipase-C. The test organism was streaked onto *Bacillus cereus* Selective Agar (BCSA) which had been supplemented with 2.5% egg yolk emulsion and incubated at 37°C for 24 hours. After incubation the plates were examined for zones of opalescence around the culture growth.

3.5.18 Aerobic cultivation of cells

A 500 ml Erlenmeyer flask containing 100 ml reconstituted infant milk formula (or a laboratory based broth medium) was inoculated with the test organism and incubated in an orbital incubator (Infors HT, Model RFI-125) at 110 rpm.

3.5.19 Glucose assay

The glucose assay procedure was carried out by the technical staff of Strathclyde Univeristy according to the standard procedure. The assay mixture was incubated at 25°C for 30 min prior to a spectrophotometrical measurement at a wavelength of 505 nm. The spectrophotometric measurement obtained was then compared to a standard glucose concentration profile (i.e. 0.02, 0.05, 0.1, 0.15, 0.20, 0.25 g L^{-1}) which had been prepared earlier, in order to establish the concentration of glucose present in the sample.

3.6 Use of the spectrophotometer

Spectrophotometric measurements are used to determine solute concentrations and to identify solutes through their absorption spectra. The theory of the instrument depends upon the application of Beer's law, which states that the extent of diminution in light intensity passing through an absorbing material depends upon the material and

concentration of the absorbing material and upon the length of the light path. This can be expressed as log $I_o/I = acl$, where I is the intensity of the beam after passing through the solution, I_o is the incident intensity, a is the extinction coefficient depending upon the particular chromogen, c is the concentration of the chromogen and l is the length of the light path through the solution. It is possible, therefore, to determine the concentration of a substance by measuring the ratio Io/I in a vessel of standard dimensions.

3.6.1 Operation of the Shimadzu spectrophotometer UV-120

The absorbance of an overnight broth culture was determined at a fixed wavelength using the Shimadzu spectrophotometer UV-120 as laid down in the manufacturers instructions.

3.6.2 Operation of the Shimadzu UV-VIS recording spectrophotometer UV-160

The Shimadzu Spectrophotometer UV-160 was operated by following the details outlined in the manufacturers "Instruction Manual" (P/N 204-04550). This spectrophotometer was primarily employed for measuring a time-course scan of absorbencies (time scan) from turbid test cultures.

3.7 Operation of centrifuges

3.7.1 Operation of the micro-centrifuge

The 1 ml samples were centrifuged (micro centrifuge, Microcentaur MSE) at the high speed setting (equivalent to 11,500 x g) for 10 minutes in a 4°C walk in refrigerator according to the manufacturers instructions.

3.7.2 Operation of the macro-centrifuge

A Model J2-21M/E (Bechman Instrument, Inc) macrocentrifuge was employed, which is a floor model refrigerated centrifuge. Operation of the J2-21M/E centrifuge was carried out following the manufacturer's instruction manual.

3.8 The Henry oblique transmitted-light technique

For over the last 40 years, the Henry oblique transillumination (OTL) technique has been employed for the initial recognition of *Listeria* colonies that appear with a distinctive bluish cast faciliting their selection in the midst of numerous other colonies (Lachica, 1990). The OTL method is a technique developed by Henry (1933) in which plates are examined under white light transmitted obliquely through the medium (BHIA) at an angle of 45°C.

The agar plates must be freed of water condensate to reduce distortion. The test organism is streaked to single colonies on BHIA and them incubated for 48 hours at 30°C. Large well isolated colonies of *Listeria* should exhibit a bluish hue primarily on the rim, whereas the bluish hue may be uniform among the small colonies on crowded sections of the plating medium.

3.9 *Bacillus cereus* enterotoxin (diarrhoeal type) test kit.

The *Bacillus cereus* Enterotoxin (diarrhoeal type) test kit (an Oxoid product TD950) was developed for the detection of *Bacillus cereus* enterotoxin (diarrhoeal type) in foods and culture filtrates by reversed phase latex agglutination (RPLA). This technique enables soluble antigen such as bacterial toxins to be detected in a simple agglutination assay. In a standard agglutination assay, soluble antibody reacts with particulate antigen such as bacterial cells. However, in a REVERSED agglutination assay the antibody, which is attached to particles, reacts with soluble antigen. The particles (in this case latex) do not themselves play a part in the reaction and they are

therefore PASSIVE. The cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible "latex agglutination" reaction.

In principle, polystyrene latex particles are sensitised with purified antiserum taken from rabbits immunised with purified *Bacillus cereus* diarrhoeal enterotoxin. These latex particles will agglutinate in the presence of *B. cereus* enterotoxin. A control reagent is provided which consists of latex particles sensitised with non-immune rabbit globulins. The test is performed in V-well microtitre plates. Dilutions of the food extract or culture filtrate are made in two rows of wells, a volume of the appropriate latex suspension is added to each well and the contents mixed. If *B. cereus* enterotoxin is present, agglutination occurs due to the formation of a lattice structure (Figure 3.5). Upon settling this forms a diffuse layer on the base of the well. If *B. cereus* enterotoxin is absent or at a concentration below the assay detection level, no such lattice structure can be formed, and a light button will, therefore, be observed.

The BCET-RPLA system was used by following the manufacturer's instructions. Food samples maybe analysed for diarrhoeal enterotoxin if an adequate extraction procedure can be put in place which will achieve a *non-turbid, fat free extract.* A dilution factor is desirable for optimum sensitivity.

3.9.1 Toxin extraction or production

Extraction from Food Matrices: A food sample (normally 25 g of infant milk power) was suspended in a sterile 500 ml Duran bottle containing 225 ml of sterile distilled water and glass beads. The contents were thoroughly mixed by shaking 25 times in 15 seconds through an excursion of 30cm. A non-turbid, fat free extract was achieved by passing the reconsitituted infant formula through a micro centrifugation (section 3.11) or a micro filtration (section 3.10) separation process. The supernatant or filtrate was subsequently filtered through a 0.2μm (millipore) low protein binding membrane filter (the filtrate was retained for assay of toxin content). Previous to this study, verification of the presence of a diarrhoeal enterotoxin in infant milk formula was achieved by confirming the presence of a *B. cereus* type 11 strain in the sample

the sample and illustrating the toxin was subsequently produced in a culture fluid (but not in the actual infant milk product).

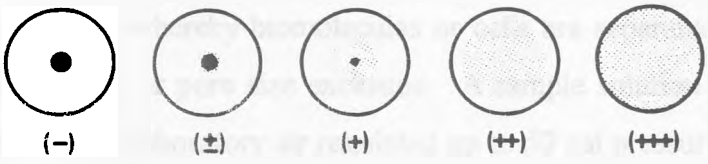
Production of Enterotoxin in Culture Fluids: the test organsim (isolated from the food sample) was inoculated into BHI broth and incubated at 35°C for 18 hours under gyrotary conditions (250 rpm). After growth, a 1 ml aliquot was transferred to a 1.5 ml Eppendorf tube and centrifuged at 11,500g for 10 mins at 4°C in a microcentrifuge. The filtrate was retained for subsequent assay of enterotoxin content. A known diarrhoeal toxin producer (*Bacillus cereus* NCTC 11145) was used as a control to check adequacy of the cultural method. Furthermore, a reconstituted toxin control TD953 (which agglutinated the sensitised latex) supplied by the manufacturer, provided a reference for the positive patterns illustrated in Figure 3.5.

3.9.2 Assay method

The assay method was carried out in accordance with the manufactures instructions.

3.9.3 Interpretation of the test results

The agglutination pattern was judged by comparison with the illustration supplied by the manufacturer:



Results classified as (+), (++), and (+++) were considered to be positive. Results in the row of wells containing latex control (TD952) should be negative. The last well in all rows should be negative and if positive patterns were observed in some of these

wells, the reaction was regarded as negative. The sensitivity of this test in detecting the diarrhoeal enterotoxin was 2ng ml^{-1} in the test extract.

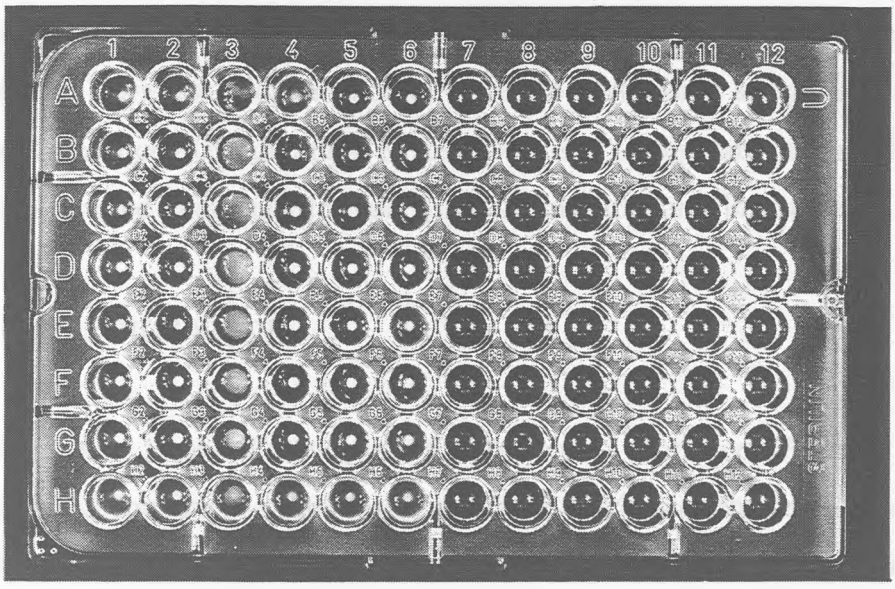


Figure 3.5 *Bacillus cereus* diarrhoeal enterotoxin production in column 3 of a V-welled microtitre plate via the Reverse Phase Latex Agglutination (RPLA) test system

3.10 Ultrafiltration

Ultrafiltration is a method whereby biomolecules or cells are separated/concentrated based on molecular weight or pore size exclusion. A sample solution was placed in the cell and nitrogen gas or laboratory air regulated up to 50 psi pressure provided the driving force for filtration. A laboratory magnetic stir plate activated the stir bar creating a gentle turbulence at the membrane surface. This caused macro-molecules to be swept off the membrane surface, and the smaller micromolecules to be drawn through the membrane (depending on whether their size is narrower than the pore dimensions of the membrane). A build-up of a dense macro molecular layer occurred at the membrane surface. The fat fraction was separated from infant milk formula by

use of the Filtron® Stirred cell Ultrafiltration System (Filtron® Technology Corporation) which had been fitted with a 10 ml disposable Omega membrane cell unit (with a nominal molecular weight limit of 300 K Daltons).

The Omega series is a polyethersulfone (PES) membrane specifically modified to minimise protein binding to the surface and interstitial structure of the membrane. This PES membrane exhibits no hydrophobic or hydrophilic interactions and is specifically designed for Ultra low protein binding, therefore maximising sample recovery. The Filtron® Stirred cell Ultrafiltration System was used to separate the milk fat fraction from reconstituted infant milk formula using the manufactures instructions.

3.11 Ultracentrifugation

The Fugisep™ 15ml Microvolume Concentrators (Intersep Filtration Systems, Berkshire,UK) consists of a filter insert which is placed inside the centrifuge tube. The sample was introduced into the filter insert which contained a membrane of the desired pore size (polyethersulphone membrane with a nominal molecular weight cut off of 100 k Daltons). The centrifuge tube was capped and the sealed tube was centrifuged (section 3.7.2). The applied g force (16,000g for 15 mins at 4°C) rapidly moved solutes and solvents of a size lower than the membrane pore size (< 100 kDa) through the filter into the outer tube. Inversion of the filter unit and recentrifugation allowed almost complete recovery of the concentrate. The concentrate was resuspended in an equivalent volume of 0.01 M PBS (15 ml) and assayed for diarrhoeal enterotoxin. (1 ml aliquot was transferred to a 1.5ml Eppendorf tube and centrifuged in a microcentrifuge (section 3.7.1) at the high setting for 10 mins). After centrifugation, the cell free liquid between the upper fat layer and the casein containing bacterial mass (bottom layer) was aspirated aseptically and tested for the presence of toxin (section 3.9). Food samples were either passed through a ultrafiltration or ultracentrifugation separation system, as aseptically aspirating the cell free liquid located between these fat and cell mass layers (obtained via microcentrifugation) cannot guarantee a fat free, non turbid extract for subsequent toxin analysis.

Operation of the Fugisep™ 15ml Microvolume Concentrator is as per the manufactures instructions (Intesep Fitration Systems, Bershire, UK). After centrifugation, the cell free liquid between the upper fat layer and the casein containing bacterial mass (bottom layer) was aspirated aseptically and tested for the presence of toxin (section 3.9).

3.12 Image analysis system

The image analysis system (Seescan Plc.) consists of a Nikon Optiphot-2 microscope, on which was mounted a Sony CCD video camera. The signal from the camera was sent to a Seescan Solitaire Image Analysis System. This then digitised the image, assigning each pixel a greyness level from 0 (white) to 256 (black), thus giving a monochrome image on the monitor.

Analysis of the image was then possible whereby the system proceeded to colour each pixel either red or blue, the colour illustrated depending on the greyness level selected by the operator (i.e. that which gave the best image definition/resolution, ensuring that subsequent measurements will have the maximum possible accuracy). The system was then able to measure objects which had been threshold (coloured). Indeed, for each feature in an image a variety of different primary measurements could be made; area, breath, perimeter, height, length, width, average feret diameter, optical density. The relevant statistical processing was then selected by the operator and performed by the image analyser.

3.13 Tyndallisation

Tyndallisation is considered as a fractional heat sterilisation process for heat labile foods (normally liquid milk products) and culture media. The test media were prepared in 500 ml pre-sterilised Duran bottles and steamed at 100°C for 30 mins on three separate days. The test media was stored under either ambient or test conditions during the intervals between each steaming process. This three day fractional

sterilisation technique heat inactivated bacterial spores present in the test media (by initially causing them to germinate by heat shocking and then destroying the emerging vegetative microbial cells by exposure to a lethal temperature).

3.14 Antagonistic assay

3.14.1 Preparation of the indicator and test organism cultures.

Prior to antagonistic assays, inocula of the indicator organisms and the test organisms were prepared. For this purpose, a single well isolated colony of the indicator organism and test organism were separately inoculated into broth media and cultivated at 30°C and/or 25°C for 18 to 24 hours. After this incubation period, the broth cultures were serially diluted by transferring 1.0 ml aliquots to 9.0 ml of sterile 0.01M PBS. Indeed, the undiluted and the diluted cultures were employed as inocula. The indicator organism and the test organism were also cultivated on agar plates and these were used when a colony was required as a source of inoculation.

3.14.2 Simultaneous or direct antagonism

3.14.2.1 Conventional method

The simultaneous or direct antagonism by the conventional method was carried out according to Batista (1993). For this experiment, 0.1 ml aliquot of an 18 hour culture of the indicator organism (and/or its decimal dilutions) was spread plated onto the surface of an agar plate. Immediately after, the test organism was seeded by stab inoculation with a straight wire through the full depth of the medium. The plates were then incubated for 24 hours at 35°C, 30°C or 25°C. After incubation, the plates were examined for the presence of a zone of clearing (inhibition zone) around the test organism.

3.14.2.2 Membrane filter technique

This technique was developed by Batista (1993) in our laboratory, and it is based on the paper-disk-plate method for determining the susceptibility of microorganisms to antibiotics. The experiment was carried out according to the following procedure: 0.1 ml of the 10^{-2} dilution of the indicator organism was spread on the surface of a TSYEA agar plate. Afterward, a membrane filter (Millipore) was placed on the surface of an seeded agar plate and 5µl of test culture was then spotted onto the membrane filter. Plates were incubated at 30°C for 24 hours. After incubation, the plates were examined for the presence of a zone of clearing or inhibition around the membrane filter.

3.14.3 Deferred antagonism

3.14.3.1 Conventional method:

Deferrred antagonism by the conventional method was performed according to the methods described by Batista (1993). The test organism was surface-inoculated onto an agar plate by the stab inoculation technique (a section of a 18 to 24 hour colony was aseptically picked up with the tip of a straight wire and stabbed throughout the depth of the medium. After seeding the test organism, the plates were incubated at 35°C, 30°C and/or 25°C for 18 to 24 hours. Following the incubation period, the surface of the agar was carefully overlaid with 5.0 ml of a soft agar (0.7% agar) which had been seeded with 0.1 ml of an 18 hour culture of the indicator organism (or a 0.1 ml aliquot taken from further decimal dilutions). These plates were then incubated for 30°C and/or 25°C for an additional 18 hours.

3.14.4 Detection of lytic bacteriophage (ϕ).

To detect the presence of lytic bacteriophage the method of Lewus et al (1991) was followed, where a portion of the clearing zone was cut from stab deferred antagonism assay plate. The agar plug was added to 3 ml of BHI broth and macerated with a sterile glass rod. The mixture was held at room temperature for 1 hour. A 100 µl amount of the suspension and 100 µl of an indicator organism (grown overnight at 30°C in BHI broth) were suspended in 4 ml of soft agar (0.7%) agar. The soft agar suspension was poured evenly over a BHI agar plate and incubated overnight at 30°C. The formation of inhibitory activity in a flip plate assay was used to rule out phage-mediated lysis.

3.14.5 Protease treatment

The protease method was carried out following the procedure of Spelhaug and Harlander (1989). Using the 10^{-3} dilution from the 18 hour indicator culture (i.e. *L. monocytogenes*), a 0.1 ml aliquot was spread plated onto a TSYEA plate. Ten ml aliquots of the 20 hour test culture (i.e. *Bacillus spp.*) were centrifuged at 11,500g for 10 mins at 4°C (3.7.1) to remove residual bacteriocin present in the broth media. The cells were then resuspended in an equal volume of TSYEB. Three enzymes, α-chymotrypsin (Sigma), trypsin (Sigma), and pronase E (Sigma) were each suspended in 0.01 M Sodium Phosphate buffer (pH 7.0) to a final cell concentration of 10 mg ml^{-1}. A 25 µl aliquot of the test cell suspension was aseptically added to 255 µl of each protease solution. A 5 µl aliquot of each protease treated cell suspension was spotted (3 spots per plate at a distance of 3 cm apart) onto the seeded TSEYA plate.
The assay plates were incubated at 30°C for an additional 18-20 hours. Protease activity was indicated on the assay plate by lack of a clear zone of inhibition around the test organism.

4 Results

4 .1 Studies on the thermal resistance and recovery of rough and smooth culture forms of *L. monocytogenes* in reconstituted infant milk formula, whole, semi-skimmed and skimmed milk and laboratory based media

4.1.1 Cultural properties of *Listeria monocytogenes*

The research program was initiated by carrying out an indepth cultural study of presumptive smooth colony forms (S-forms), typical of standard *Listeria monocytogenes* strains. The *Listeria* strains were obtained from the National Collection of Type Cultures (NCTC), Colindale, London (section 3.1.1). In order to become aufait with the typical morphological, physiological and biochemical characteristics of *Listeria monocytogenes* strains and to become confident about future identification of presumptive *Listeria* species a two part cultural study was undertaken.

Firstly, a detailed study was performed on the general morphological (cell and colony form), physiological and biochemical properties of a variety of smooth *L. monocytogenes* test strains, i.e. this enabled the identification of typical cultural properties and also facilitated familiarisation with the unique set of characteristics.

Secondly, since the morphological appearance (cell and colony) of *L. monocytogenes* is a key issue in this research, the performance and morphological characteristics of different *L. monocytogenes* strains were assessed on a variety of cultural plating media (i.e. both non selective and selective media).

During the course of this research, a variety of pleomorphic surface culture forms of *L. monocytogenes* (designated as rough form) were identified. These rough colony forms (R-form) are culture variants of the smooth colony form mentioned earlier and differed from these parent S-forms in either size, texture, elevation, consistency, configuration, opacity, colour, morphology, biochemical reaction, degree of

haemolysis and virulence (section 2.1.10). In order to compare the cell and morphological characteristics of the smooth and rough colony forms of *L. monocytogenes*, the key morphological, physiological and biochemical properties of R-form culture variants (derived from subsequent research carried out in section 4.2) were examined in this section.

4.1.1.1 Properties of the smooth and rough culture forms of *Listeria monocytogenes*.

The study comprised of a detailed cultural analysis of three presumptive smooth and rough colony forms of *L. monocytogenes*. The three smooth *Listeria* test cultures were obtained from the NCTC and were designated the following test numbers to facilitate these cultural studies: (1) = *Listeria monocytogenes* 4b (NCTC 11994); (2) = *Listeria monocytogenes* 4b (NCTC 9863) and (3) *Listeria monocytogenes* 1a (NCTC 10357).

Over the course of subsequent thermal inactivation studies (section 4.3), rough colony forms of *L. monocytogenes* were generated from each of the above mentioned smooth (typical) colony forms. These R-form colonies were assigned the following test number: (4) *Listeria monocytogenes* 4b (NRB5, a culture variant of NCTC 11994); (5) *Listeria monocytogenes* (NRB2, a culture variant of NCTC 9863) and (6) *Listeria monocytogenes* 1a (NJ2, a culture variant of NCTC 10357).

Single, well isolated colonies of the 6 test strains of *L. monocytogenes* were grown on a non selective (TSYEA) and selective (LSA-Oxford formulation) plating medium over a 24 and 48 hour period at 35°C and subsequently examined for the following morphological (i.e. cell and/or colony) characteristics: shape, size and arrangement of cells; shape, size, outer margin and surface appearance of colony; colony elevation, colour, opacity and configuration.

In addition to this specific culture study (i.e. involving 6 test strains), this section also revealed other variations in the cell and colony characteristics exhibited by atypical surface cultures throughout the course of this research (section 4.1.3).

As can be observed from Table 4.1, the cell morphology of the three smooth strains of *L. monocytogenes* (i.e. numbers 1 to 3 inclusive) consisted of very short Gram positive rods (or coccobacilli) which were arranged singly (0.4 - 0.5μm in diameter and 1.0 - 2.0 μm in length), paired and sometimes the cells were arranged in a V shape (No.2). The composition of the plating medium did not influence cell shape arrangement. Apart from a variable Gram stain reaction for each of the three smooth test strains, no change in cell shape or arrangement occurred after an additional 24 hours storage at 35°C.

The cell morphology of the rough form test strains (No. 4 and 5) differed from the above S-from after 24 hours at 35°C, as they frequently demonstrated short to long chains (up to 45 μm in length), in addition to the single (0.8 to 2.0 μm) and paired cell arrangement. Closer examination of the Gram stains revealed the apparent abscence of double septae separating individual cells within these short to long chains. Further studies to demonstrate the existance of septae were not persued. Test strain No. 6 showed similar cell properties to the standard smooth form mentioned above.

Table 4.1 Cell morphology characteristics of *L. monocytogenes* which had been cultivated on either TSYEA or LSA plates for 24 hours at 35°C.

Listeria **Test Strain Number**[a]	**Cell Morphology**					
	Gram Stain Reaction		**Cell Shape**		**Cell Arrangement**	
	TSYEA[b]	**LSA**	**TSYEA**	**LSA**	**TSYEA**	**LSA**
1	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single or paired	Single or paired
2	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single or paired	Single or paired
3	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single or paired	Single or paired
4	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single, paired, chains	Single, paired, chains
5	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single, paired, chains	Single, paired, chains
6	+	+	Short rods (0.5 x 2μm)	Short rods (0.5 x 2μm)	Single or paired	Single or paired

[a] refers to the *L. monocytogenes* test strain number in setion 4.1.1
[b] refers to the plating media

An additional 24 hours storage at 35°C resulted in Gram variable reactions for each of the three R form test strains (i.e. No. 4 to 6). An example of long chain formation is illustrated in Figure 4.1, where *L. monocytogenes* (No.4) was cultivated on TSYEA for 24 hours at 35°C prior to analysis. Moreover, as test strain No.6 did not exhibit any chain formation, it would appear that the presence of a long chain cell arrangement is not a prerequisite for the subsequent development of rough colonies. The composition of the plating medium did not effect the cell shape or arrangement of R-form *L. monocytogenes* cells, as similar morphological characteristics were observed for each of these test strains on TSYEA and LSA plating media.
According to Bergey's Manual of Systematic Bacteriology (Seeeliger and Jones 1986), the morphological cell characteristics of the R- type test strain number 4 and 5 do not conform to the morphological properties of the genus *Listeria* as long cellular chain formations are not typical of young cultures.

4.1.1.2 Colonial characteristics of *Listeria monocytogenes* test strains

A single well isolated colony of each of the *L. monocytogenes* strains mentioned in section 4.1.1 was obtained on TSYEA and LSA (Oxford formulation) plates via the spread plate technique. These plates were incubated at 35°C for 24 and 48 hours. After each 24 hour observation period, the resulting colonies were examined for the following morphological characteristics: shape, size, appearance of outer margin (i.e. edge), surface texture, elevation and colour. Additional morphological and physiological culture tests were performed, such as: the appearance of presumptive *L. monocytogenes* colonies under Henry's Oblique Transmitted Light (OTL) (section 3.8), haemolytic reaction on horse blood agar (section 3.5.6), CAMP test reaction on sheep blood agar (section 3.5.7), tumbling motility (section 3.5.10) and cytochrome oxidase activity (section 3.5.9). A biochemical profile analysis of these R and S- forms was carried out using the commercially available biochemical galleries (i.e. API *Listeria*, API- CORYNE and MICRO ID *Listeria*). The section was concluded with a comparison of both surface culture forms which developed on various plating media over a 48 hour period via the image analysis technique (section 3.12).

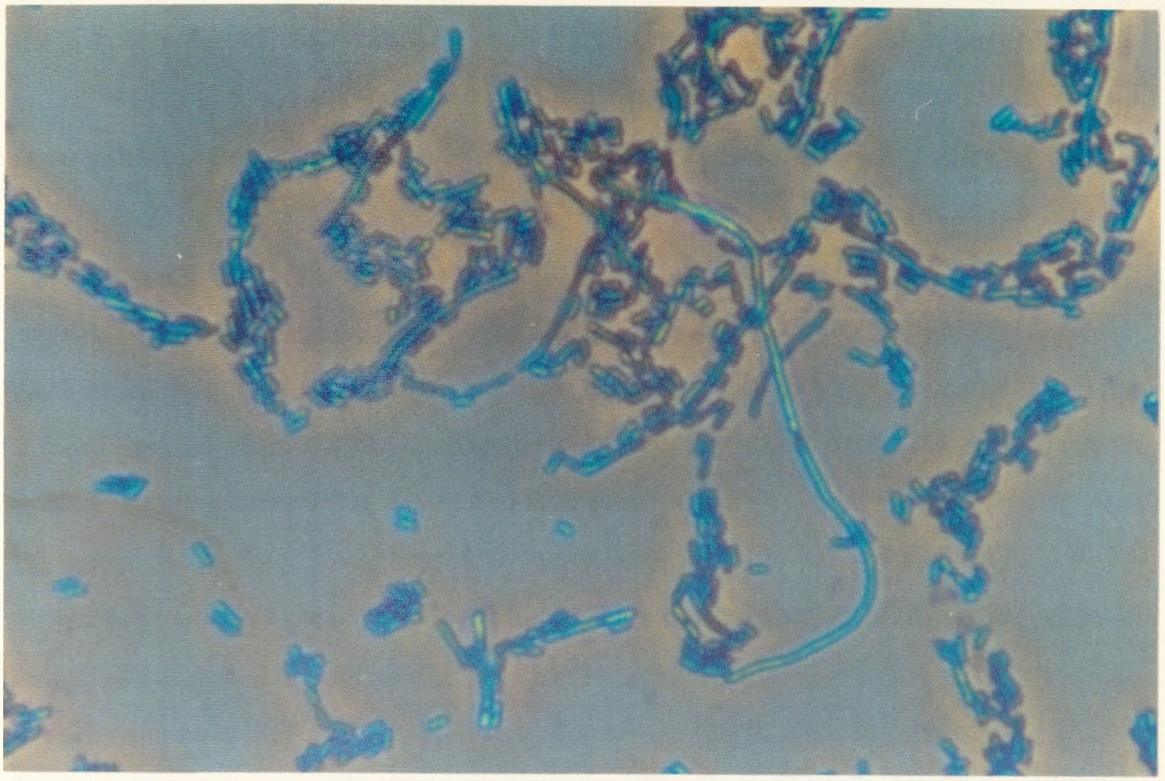


Figure 4.1 *L. monocytogenes* (NJ2) exhibiting a single, paired and filimentous cellular arranged after 24 hours growth on TSYEA at 37°C.

The 3 "smooth" forms of *L. monocytogenes* (numbers 1 to 3) exhibited similar colonial characteristics on TSYEA plates after 24 and 48 hours growth at 35°C. After 24 hours growth of these test strains on this non-selective plating media, the surface cultures were circular in shape, approximately 1.0-1.5 mm in diameter, entire outer margin, low convex elevation, translucent in colour with smooth surface appearance (some isolated colonies exhibited surface papillation - which appeared in clusters or in a straight line traversing the surface). After an additional 24 hours cultivation, these S-form colonies showed the same morphological properties commensurate to the increase in cell size, which ranged from 2.5 to 5.0 mm in diameter.

While these smooth test strains exhibited similar colonial properties on the LSA plates after 24 and 48 hours incubation at 35°C, the resulting surface cultures differed from the S-form colonies when examined on TSYEA plates. Indeed, after 24 hours growth on LSA plates, the colonies (generated from strains 1 to 3) were circular in shape, approximately 1mm in diameter, having an entire outer margin, elevation varying from a flat plateau to umbilligate (i.e. a black circular depression at the centre of the colony), grey in colour between the outer margin and the edge of this circular depression and non granulated. A similar morphological appearance was observed after 48 hours growth on the LSA plates. However, as the colony size increased to 2.5-3.0 mm in diameter, a commensurate increase in the central depression was evident, ranging from 0.5 to 1.5 mm in diameter.

In terms of colony morphological characteristics, the 3 rough strains of *L. monocytogenes* (numbers 4 to 6) differed from each other (and from the smooth colony form) on TSYEA and LSA culture media. After 24 hours cultivation on TSYEA plates at 35°C, these pleomorphic colony forms were approximately 1.0 to 2.0 mm in diameter; with either an irregular (No.4), scalloped (No.5) or crenated (No.6) outer margin; translucent (No.4 and 6) to opaque (No.5) in colour; convexed (No.4 and 5) to umbilligate (No.6) in elevation; with or without surface papillation (clustered or linear arrangements). The colony characteristics exhibited after 24 hours persisted at 48 hours growth, with the colonies increasing in size from 2.5 to 5.5 mm in diameter.

Generally, rough colony forms which had developed on the TSYEA plates after 24 and 48 hours growth, following a sublethal heat treatment (section 4.1.2.2.1), differed from each other and from the standard smooth surface culture form: in size (1.0 to 2.0 mm and 2.5 to 6.5 mm in diameter after 24 hours and 48 hours growth respectively), outer margin (either smooth, crenated. scalloped, undulated or irregular), elevation (domed, convexed, umbilligated, umbonated, or low convexed), colour (translucent to opaque) and shape (either circular, oval or irregular).

Furthermore, some R-forms demonstrated the presence of an inner margin which took the general shape of the colony. However, pleomorphic colonies which exhibited an entire, scalloped, crenated or irregular outer margin nearly always showed a circular

inner margin (where present). Certain rough colony forms exhibited surface papillation. The presence of long cell chains was not a prerequisite for rough colony development, as not all pleomorphic colony variants exhibited chain formation.

The rough colony forms (test strain numbers 4 to 6) of *L. monocytogenes* illustrated dissimilar colony characteristics compared to those of the above mentioned "smooth" colony form on LSA plates after 24 hours at 35°C. These surface culture variations include: shape (either irregular{No.4 and 5) to oval {No.6}); outer margin (irregular {No.4), scalloped {No.5) to slightly undulated {No.6}). While these R-colony forms shared certain similar characteristics, such as elevation (umbilligate to plateau), size (1.0 to 2.0 mm), colour (grey outer margin with a black central depression).

With the exception of colony size (some of which measured 5.5 mm in diameter), the same colonial characteristics were identified in 48 hour old cultures. However, some R-forms exhibited surface papillation (either scattered, clustered or arranged in a straight line across the surface). Generally, both presumptive S and R test strains of *L. monocytogenes* (numbers 1 to 6) illustrated a characteristic bluish tinge when examined by oblique transmitted light.

4.1.1.3 Complimentary tests.

(A1) Haemolysis: Single colonies of each *L. monocytogenes* (S and R colony types) cited in section 4.1.1.1, were analysed for the ability to haemolyse red blood cells (i.e. β-haemoytic reaction). The test was performed as described in section 3.5.6.

A variable haemotytic activity was observed by the 6 *L. monocytogenes* test strains, where strains 1, 2, 4 and 5 exhibited weak β-haemolysis and strains 3 and 6 (i.e. culture variants of *L. monocytogenes* 1a, NCTC 10357) failed to show any haemolytic activity.

(A2) Tumbling Motility: Both the so called smooth and rough presumptive culture forms of *L. monocytogenes* (test strains 1 to 6 inclusive) were analysed for the ability to move in a characteristic tumbling or 'head over heels' motility pattern. Confirmation of this tumbling motility was achieved by initially cultivating the test

strains in BHI broth at 25°C for 24 hours prior to examination of a hanging drop preparation (section 3.5.10).

The test illustrated that all 6 *Listeria* test strains demonstrated this characteristic tumbling motility when examined in the hanging drop preparation under phase contrast. Moreover, the long chains present in certain rough surface cultures (e.g. test strain 4 and 5) also exhibited this tumbling motility pattern, but to a lesser extent compared to that of single and/or paired *L. monocytogenes* cells. This impairment of motility was possibly due to the restrictions imposed on the chains in the hanging drop preparation (i.e. as single and/or paired *Listeria* cells appeared to have more freedom of movement within the microscopic field of view).

(A3) CAMP Test: The CAMP test assists differentiation between members of the genus *Listeria* (and helps to distinguish between members of the *Listeria* genus and other bacterial genera) by illustrating whether or not certain *Listeria* species exhibit an augmented haemolytic activity on sheep blood agar, when the cells are grown in close proximity to either *Staphylococcus aureus* (i.e. a strong β-haemoytic producing strain) and *Rhodococcus equi*. This procedure was carried out according to the procedure described in section 3.5.7.

The CAMP test revealed that *L. monocytogenes* test strains 1, 2, 4, and 5 demonstrated an enhanced area of β-haemolysis at the point where the *Listeria* test strains had grown in close proximity to that of the *S. aureus* streaked line. However, this β-haemolytic enhanced activity was not evident for the remaining *L. monocytogenes* test strains (i.e. test strain number 3 and 6).

Indeed, it was observed during the course of this research that repeated CAMP tests employing the same test strains resulted in a generalised decrease in the zone of clearing (β-haemolysis) near the *S. aureus* line. This reduction in β-haemolytic intensity was due to the diminished ability of *S. aureus* to elicit an enhanced β-haemolytic reaction on repeated subcultures.

(A4) Cytochrome Oxidase: The oxidase test was carried out in accordance with the procedure described in section 3.5.9. The oxidase contact sticks illustrated a negative reaction (remained pink in colour) for all 6 *L. monocytogenes* test strains.

4.1.1.4 Identification of presumptive *Listeria monocytogenes* using biochemical test kits

The biochemical characteristics of *L. monocytogenes* strains (numbers 1 to 6) were evaluated using three commercially available microbiological test kits, such as: API *Listeria* and API-CORYNE, and MICRO-ID *Listeria* (section 3.5.8.5).

(B1) API *Listeria*: the biochemical test results for the smooth and rough forms of *L. monocytogenes* are illustrated in Table 4.2. As can be seen from Table 4.2, the culture variants of *L. monocytogenes* 1a (i.e. No.3 and 6) gave a positive result for the DIM test (this test is 99% positive for *L. innocua* strains and only 1% positive for *L. monocytogenes* strains), which would imply that these test strains of *L. monocytogenes* are incorrectly classified as *L. innocua*. The remaining test strains (No.1, 2, 4 and 5) were correctly identified as *L. monocytogenes*.

(B2) API-CORYNE: according to these API-CORYNE test results, strain numbers 3 and 6 (i.e. culture variants of *L. monocytogenes*) exhibited 3 tests (i.e. pyrazinamidase, N-acetyl-β-glucosaminidase and sucrose utilisation) which were atypical of the biochemical characteristics for *L. monocytogenes* (Table 4.2). Classification was obtained to the *Listeria* genus only, with no assignment made to *Listeria* species level. While test strain numbers 1, 2, 3 and 4 were classified as either *L. monocytogenes* or *L. innocua*, although these *Listeria* strains exhibited a negative pyrazinamidase test which is uncharacteristic for *L. monocytogenes*.

Table 4.2. Idenitification of *Listeria monocytogenes* via API-*Listeria*, API-CORYNE and MICRO-ID *Listeria* galleries, in addition to complimentary tests.

Test	Smooth Morphological Form			Rough Morphological Form		
	Strain No.1	Strain No.2	Strain No.3	Strain No.4	Strain No.5	Strain No.6
Tumbling Motility	+	+	+	+	+	+
β-Haemolysis	+	+	-♣	+	+	-♣
Camp Test- S.aureus	+	+	-♣	+	+	-♣
- R.equi	-	-	-	-	-	-
Cytochrome Oxidase	-	-	-	-	-	-
API Listeria						
DIM	-	-	+♣	-	-	+♣
Esculin	+	+	+	+	+	+
α-Mannitol	+	+	+	+	+	+
D-Arabitol	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-
Rhamnose	+	+	+	+	+	+
α-Methyl-D-Glucoside	+	+	+	+	+	+
Ribose	-	-	-	-	-	-
Glucose 1-Phosphate	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-
β-Haemolysis	+	+	-	+	+	-
API-CORYNE						
Nitrate Reduction	-	-	-	-	-	-
Pyrazinamidase	-♣	-♣	-♣	-♣	-♣	-♣
Pyrrolidonyl Arylamidase	-	-	-	-	-	-
Alkaline Phosphate	-	-	-	-	-	-
β-Glucuronidase	-	-	-	-	-	-
β-Galactosidase	-	-	-	-	-	-
N-Acetyl-β-Glucosaminidsae	+	+	-♣	+	+	-♣
Esculin	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Gelatine hydrolysis	-	-	-	-	-	-
0 (Control fermentation)	-	-	-	-	-	-
Glucose	+	+	+	+	+	+
Ribose	-	-	-	-	-	-
Xylose	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Lactose	-	+	-	-	+	-
Sucrose	-	-	-	-	-	-
Glycogen	-	-	-	-	-	+♣
Catalase	+	+	+	+	+	+
MICRO-ID Listeria						
Acetoin Production (VP)	+	+	+	+	+	+
Nitrate Reduction	-	-	-	-	-	-
Phenylalanine Deaminase	-	-	-	-	-	-
Hydrogen Sulphide	+	+	+	+	+	+
Indole Production	-	-	-	-	-	-
Ornithine Decarboxylase	-	-	-	-	-	-
Malonate	-	-	-	-	-	-
Urease	-	-	-	-	-	-
Esculin	+	+	+	+	+	+
β-Galactoside	-	-	-	-	-	-
Xylose	-	-	-	-	-	-
Rhamnose	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-

♣ test results which appear incorrect for *L. monocytogenes* identification

(B3) MICRO-ID *Listeria*: the results of these biochemical and/or fermentation tests revealed that strain numbers 3 and 6 (i.e. culture variants of *L. monocytogenes* 1a) were classified as belonging to the genus *L. innocua* (Table 4.2). Indeed, while test strains 3 and 6 illustrated positive reactions for all the biochemical test for *L. monocytogenes*, they did not give a positive CAMP test reaction (this CAMP test is an important test to assist the differentiation between *L. monocytogenes* and *L. innocua*, via the MICRO-ID *Listeria* system). The remaining test strains of presumptive *L. monocytogenes* (numbers 1, 2, 4 and 5) were correctly identified as *L. monocytogenes* using this MICRO-ID *Listeria* test system.

(A4) API 50 CHB and API 20 E: The API 50 CHB (*Bacillus*) kit (Biomerieux) is supplemented with the first 12 tests of the API 20 E (Enterobacteriaciae) for the subsequent identification of *Bacillus* species. The API 50 CH (which is made up of 49 different dehydrated carbohydrates) and API 20 E kits were employed to discover whether the rough colony forms of presumptive *L. monocytogenes* exhibited a different biochemical and/or sugar fermentation profile compared to their parent smooth colony forms (Table 4.3).

While all three smooth forms of *L. monocytogenes* (test strain numbers 1 to 3) exhibited a similar sugar fermentation (on API 50 CHB gallery) and biochemical (on API 20 E) profiles, the rough forms differed slightly in sugar fermentation ability compared to their parent smooth strains. Test strain number 4 (*L. monocytogenes* 4b) differed from its parent strain by demonstrating a positive reaction for melezitose ulilisation.

While the pleomorphic test strain numbers 5 and 6 also differed from their parent *L. monocytogenes* strains by illustrating a negative reaction for salicine fermentation (strain number 5) and a positive reaction for sorbitol and glycogen utilisation (strain number 6) respectively.

Table 4.3. Biochemical analysis of pleomorphic forms of *Listeria monocytogenes* via API 50 CHB and API 20 E galleries.

Test	Smooth Morphological Form			Rough Morphological form		
	Strain No.1	Strain No.2	Strain No.3	Strain No.4	Strain No.5	Strain No.6
API 50 CHB						
Control	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-
Ribose	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-
β-Methyl-Xyloside	-	-	-	-	-	-
Galactose	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-
Rhamnose	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-
Inositol	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	+♥
α-Methyl-D-Mannoside	+	+	+	+	+	+
α-Methyl-D-Glucoside	+	+	+	+	+	+
N-Acetyl-Glucosamine	+	+	-	+	+	-
Amygdaline	+	+	+	+	+	+
Arbutine	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
Salicine	+	+	+	+	-♥	+
Cellobiose	+	-	+	+	+	+
Maltose	+	-	+	+	+	+
Lactose	+	-	+	+	+	+
Melibiose	-	-	-	-	-	-
Saccharose	+	-	+	+	+	+
Trehalose	+	-	+	+	+	+
Inuline	-	-	-	-	-	-
Melezitose	-	-	-	+♥	-	-
D-Raffinose	+	-	+	+	+	+
Amidon	+	-	+	+	+	+
Glycogen	-	-	-	-	-	-♥
Xylitol	+	-	+	+	+	+
β-Gentiobiose	+	-	+	+	+	+
D-Tyranose	+	-	+	+	+	+
D-Lyxose	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-
D- Arabitol	+	-	+	+	+	+
L-Arabitol	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-
2-ceto-Gluconate	-	-	-	-	-	-
5-ceto-Gluconate	-	-	-	-	-	-

♥ test results which differ from the parent "smooth" strain of *Listeria monocytogenes*.

Table 4.3 continued.

TesT	Smooth Morphological Form			Rough Morphological Form		
	Strain No.1	Strain No.2	Strain No.3	Strain No.4	Strain No.5	Strain No.6
API 20E						
β-Galactosidase	-	-	-	-	-	-
Arginine Dihydrolase	-	-	-	-	-	-
Lysine Decarboxylase	-	-	-	-	-	-
Ornithine Decarboxylase	-	-	-	-	-	-
Citrate Utilisation	-	-	-	-	-	-
Hydrogen Sulphide	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Tryptophane Deaminase	-	-	-	-	-	-
Indole Production	-	-	-	-	-	-
Acetoin Production	+	+	+	+	+	+
Gelatin Production	-	-	-	-	-	-
Nitrate Production	-	-	-	-	-	-

4.1.1.5 The development of rough and smooth colony forms of *L. monocytogenes* over a 48 hour cultivation period at 25°C, as examined and measured via the Seescan® Image Analysis technique.

The aim of the study was to follow the cell and colony morphological development of a rough colony form of *L. monocytogenes* (NRB2) on solid plating media by way of measuring (viz Image Analysis program) the change in colony appearance over a 24 and 48 hour cultivation period. Determination of the rate of morphological change was achieved by comparing the development of the rough *Listeria* surface culture against the growth of a smooth colony form (NCTC 11994) under the same cultivation conditions.

Quantification of the observed morphological change was performed via cell and colony measurements which were obtained after a 24 and 48 hour growth period from non-selective (TSYEA) and selective (LSA) plating media. The cell/colony dimensions (i.e. from both morphological forms of *Listeria*) were measured and analysed using the Seescan Image Analysis program (section 3.12), while photographs of the cell arrangement and the appearance of the developing colony were taken at each sample time period.

By cultivating both the smooth and rough colony forms of *L. monocytogenes* on solid media under the same set of culture conditions, it was proposed that these specific morphological measurements would establish whether these pleomorphic surface culture forms were significantly different (at the P0.05 level via Mann- Whitney U-test). In order to investigate the above, the following experimental line of approach was adopted.

Single colonies of *Listeria monocytogenes* NCTC 11994 (i.e. smooth colony type) and *L. monocytogenes* (NRB2 i.e. rough colony type) were obtained on TSYEA plates after 24 hours storage at 35°C using the streak plate technique.

A representative colony from each morphological type was inoculated into separate 250 ml Erlenmeyer flasks containing 100 ml TSYEB, which were then incubated in an orbital incubator at 110 rpm for 24 hours at 37°C. After this 24 hour growth period, a serial decimal dilution (from 10^{-8} to 10^{-4}) of the respective test cultures were performed by aseptically transferring 1ml of culture into 9ml 0.01M PBS (section 3.3.1). A 0.1 ml aliquot 10^{-4} dilution was aseptically transferred into a separate set of 250 ml Erlenmeyer flasks containing 100 ml of sterile TSYEB (i.e this was repeated for both types of organisms). The test cultures were cultivated by agitating at 110 rpm for 24 hours at 37°C to ensure that a homogeneous distribution of cells occurred in the culture media. A decimal dilution series was made of the respective 24 hour test cultures as described above.

There after, a 0.1 ml aliquot of the 10^{-8} dilution (and each dilution series down to and including 10^{-4}) was spread plated in duplicate onto two sets of TSYEA and LSA plates (6 plates used at each dilution). One set of seeded *Listeria* plates was incubated at 30°C for 24 hours, while the other was grown at 25°C. This was done in order to identify the most suitable cultivation temperature for the generation of small measurable surface colonies (i.e to determine the incubation temperature which would provide small measurable colonies from both pleomorphic colony types).

The Seecan Image Analysis program (section 3.12) was programmed to determine colony parameters such as area (μm^2), perimeter (μm), feret diameter (i.e. the mean diameter of colonies taken from multiple measurements), length/ breadth ratio and area/perimeter ratio's for 20 randomly selected colonies which had developed on the

respective TSYEA and LSA plates after 24 hours. These R and S-form colony measurements were obtained by transmitting the field of view observed from the light microscope (x4 objective) onto the screen of the Seescan Image Analyser. The results of these measurements are shown in Table 4.4 and Figures 4.2 to 4.9 inclusive.

A Gram stain was performed on each type of test surface culture and the cell arrangement was viewed and measured under the x100 objective. Accurate measurement (μm) of these cell arrangements (n=30) were obtained by means of the image analyser program

The plates containing 24 hours growth were then re-incubated for an additional 24 hours at 25°C, where upon further cell and colony measurments were made as described above. Due to the limitations imposed by the light microscope (at the lowest objective of x4), it was not possible to photograph some of the larger colonies as they were outside the field of view of the 35 mm camera. This appeared to be the case for certain S and R surface culture forms which had grown on the TSYEA plates, whereas colonies which had developed on the selective plating medium (LSA) were smaller in area and diameter and could be photographed in entirety.

However, as the image analyser (x4 objective) was connected to a computer monitor, this provided a slightly wider field of view compared to the limited frame of the 35mm camera, thus enabling all resulting S and R form colonies which had developed on both plating media after 24 and 48 hours to be imaged and measured. Results showed that smaller measurable colony forms emerged when both the R and S-form were cultivated at the reduced temperature of 25°C.

The average of 30 measurements from randomly selected colonies are illustrated in Table 4.4. Statistical analysis (i.e. Mann-Whitney U test) of the information supplied in this table has shown that there was no significant difference between the 30 sample measurements for the same morphological form cultivated on TSYEA and/or LSA plates over the 48 hour period. However, the S-form cultivated on TSYEA plates for 24 hours at 25°C (Figure 4.2) was significantly different (P 0.05) from the S- form cultivated for 24 and 48 hours on LSA plates (Figures 4.6 and 4.8), the S-form cultivated on the same plating media for 48 hours (Figure 4.4) and from the R-form

cultivated for 24 and 48 hours on both TSYEA (Figures 4.3 and 4.5) and LSA plates (Figures 4.7 and 4.9).

The rough surface culture measurements (for all morphological parameters) obtained after 24 hours growth on TSYEA plates (Figure 4.3) were significantly different (P0.05) from those obtained at the same observation period on LSA plates (Figure 4.7, from the R-form measurements obtained after an additional 24 hours incubation on both plating media (Figures 4.5 and 4.9), and compared to the S-form surface culture measurements obtained after 24 and 48 hours on TSYEA (Figures 4.2 and 4.4) and LSA plates (Figures 4.6 and 4.8). These differences in colony appearance were shown to be significant (P0.005) for all the morphological parameters employed (Table 4.4).

Table 4.4 Colony measurements obtained for both rough and smooth forms of *L. monocytogenes* cultivated on TSYEA and LSA plates over a 24 and 48 hour period at 25°C.

Colony Form	Growth Conditions	Area (μm^2)	Perimeter (μm)	Feret Diameter	Length/ Breath Ratio	Area/ Perimeter Ratio
Smooth	24h (TSAYE)[i]	$6.38x10^4$	916.2	287	1.096	68.87
Smooth	24h (LSA)[ii]	$3.90x10^4$	722.3	226	1.177	52.71
Rough	24h (TSAYE)	$1.65x10^3$	201.2	525	1.261	81.22
Rough	24h (LSA)	$1.56x10^4$	452	141.2	1.157	32.52
Smooth	48h (TSAYE)	$9.45x10^4$	3563	Not Sampled	.098	2654
Smooth	48h (LSA)	$8.17x10^5$	3295	Not Sampled	1.092	247.8
Rough	48h (TSAYE)	$1.01x10^6$	5012	Not Sampled	1.272	201.7
Rough	48h (LSA)	$1.17x10^6$	4306	Not Sampled	1.138	270.7

[i] illustrates the mean of 30 meansuremnts from surface cultures which emerged on TSYEA plates after 24 hours incubation.
[ii] illustrates the mean of 30 meansuremnts from surface cultures which emerged on LSA plates after 24 hours incubation.

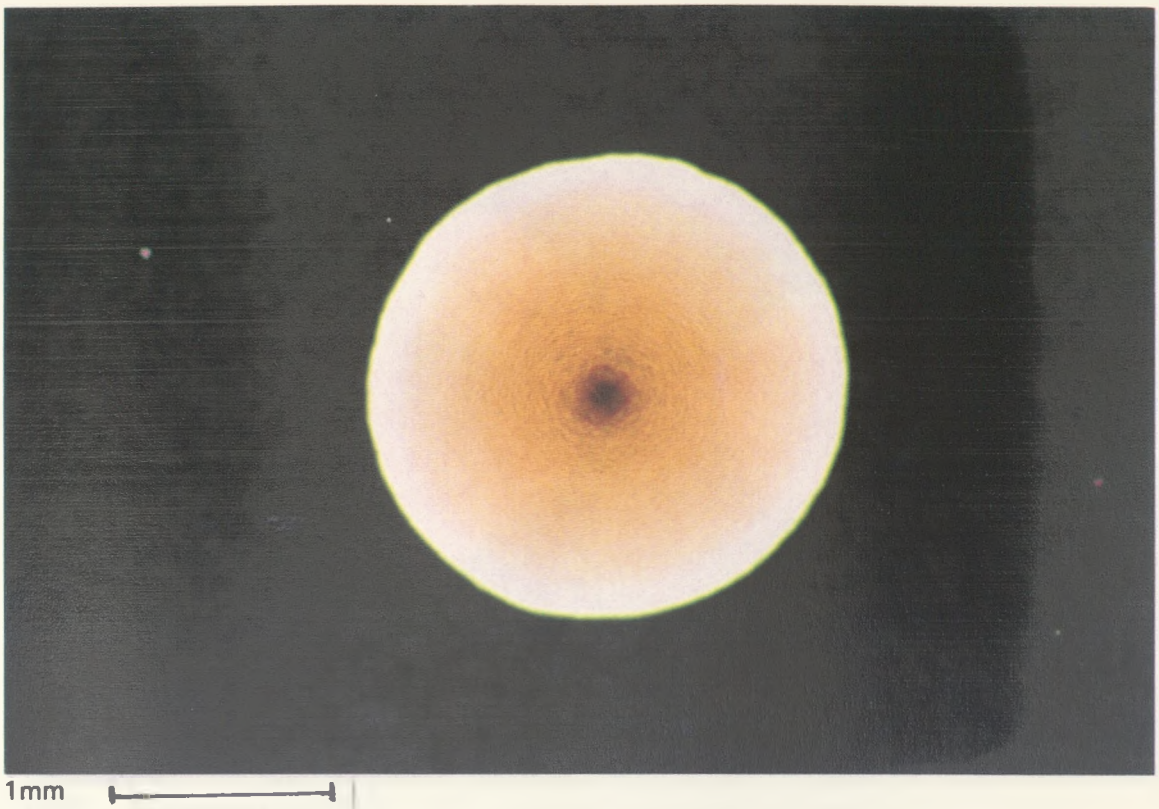
The cell length (μm) and arrangement for the S-form surface cultures were not significantly different, consisting of a homogenous distribution of either single (1.5 μm in length) and/or paired (3.0 - 3.2 μm in length) cells. The cell composition (i.e. arrangement and dimensions) of the R-form differed from the S-form after 24 and 48

hours growth on TSYEA (Figures 4.3 and 4.5) and LSA (Figures 4.7 and 4.9) plating media, by being composed of single, paired and long cell chain arrangements (up to 60 μm in length). These characteristic cell arrangements shown in these figures were the result of Gram stain reactions.

4.1.2 Identification of key cultural conditions which will optimise the recovery of heat stressed *Listeria* cells from contaminated infant milk formulae.

As described in section 2.3.1, the manufacture of infant milk formulae (IMF) involves the use of process temperatures which are normally bactericidal to non-spore forming microorganisms. Considering the ubiquitous nature of *Listeria monocytogenes* and the susceptibility of infants to listeriosis, an indepth bacteriological analysis of commercially available IMF for this opportunist pathogen was deemed to be warranted. In order to properly screen these dehydrated infant powders for uninjured and/or sublethally injured *Listeria* cells, it was necessary to investigate and identify key growth, heating, diluting, enriching and plating conditions which would favour the resuscitation of these heat stressed cells.

Consequently, the early series of experiments focused on generating a known, uniform concentration of *Listeria* cells (CFU ml^{-1}) which were both homogeneous in cell number and the stage reached in their growth cycle. The following series of studies involved; identifying, implementing and modifying cultural conditions previously shown to enhance the thermal resistance of non-spore forming bacterial cells (section 2.1.3); determining and optimising the heat resistance of different strains and morphological colony forms of *Listeria monocytogenes* in a variety of culture media under these optimised cultivation, heating and recovery conditions; identifying the most suitable resuscitation broth and recovery conditions for the subsequent detection of heat treated *Listeria* cells in IMF, and determining whether the efficacy of standard recommended recovery methods, such as the Food and Drug Administration



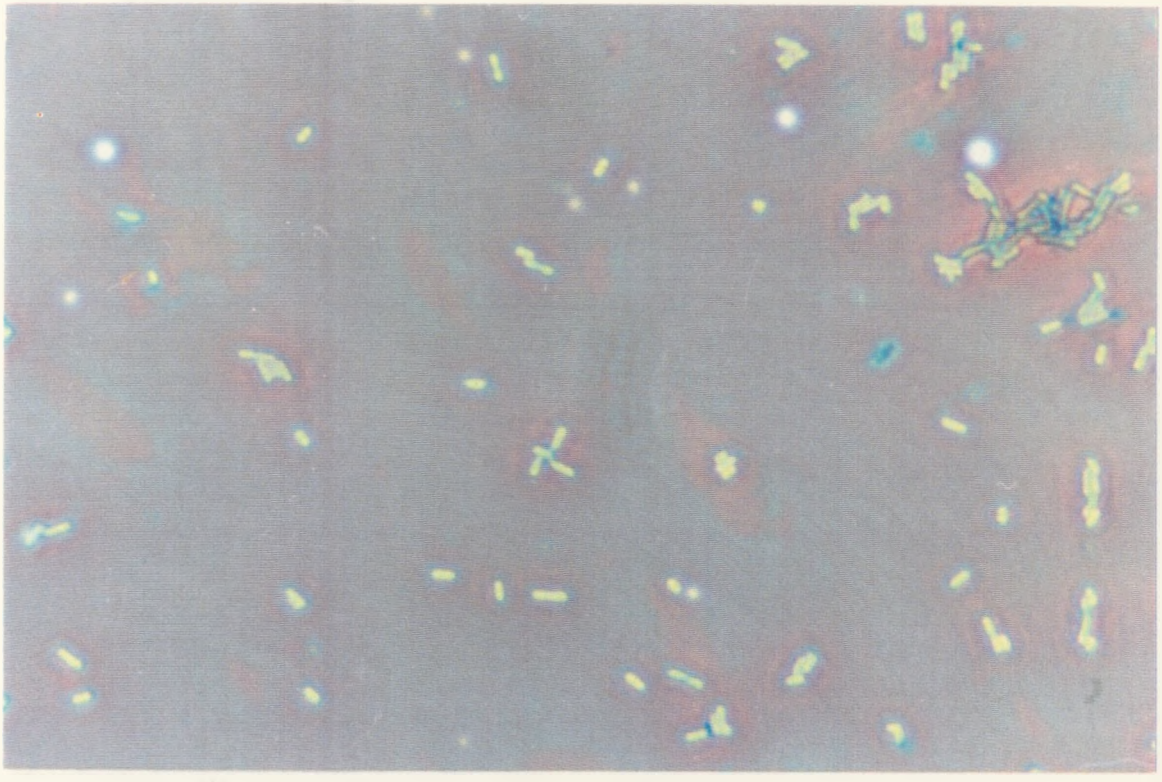


Figure 4.2 Growth of smooth form of *L. monocytogenes* (NCTC 11994) on TSYEA plates after 24 hours at 25°C.

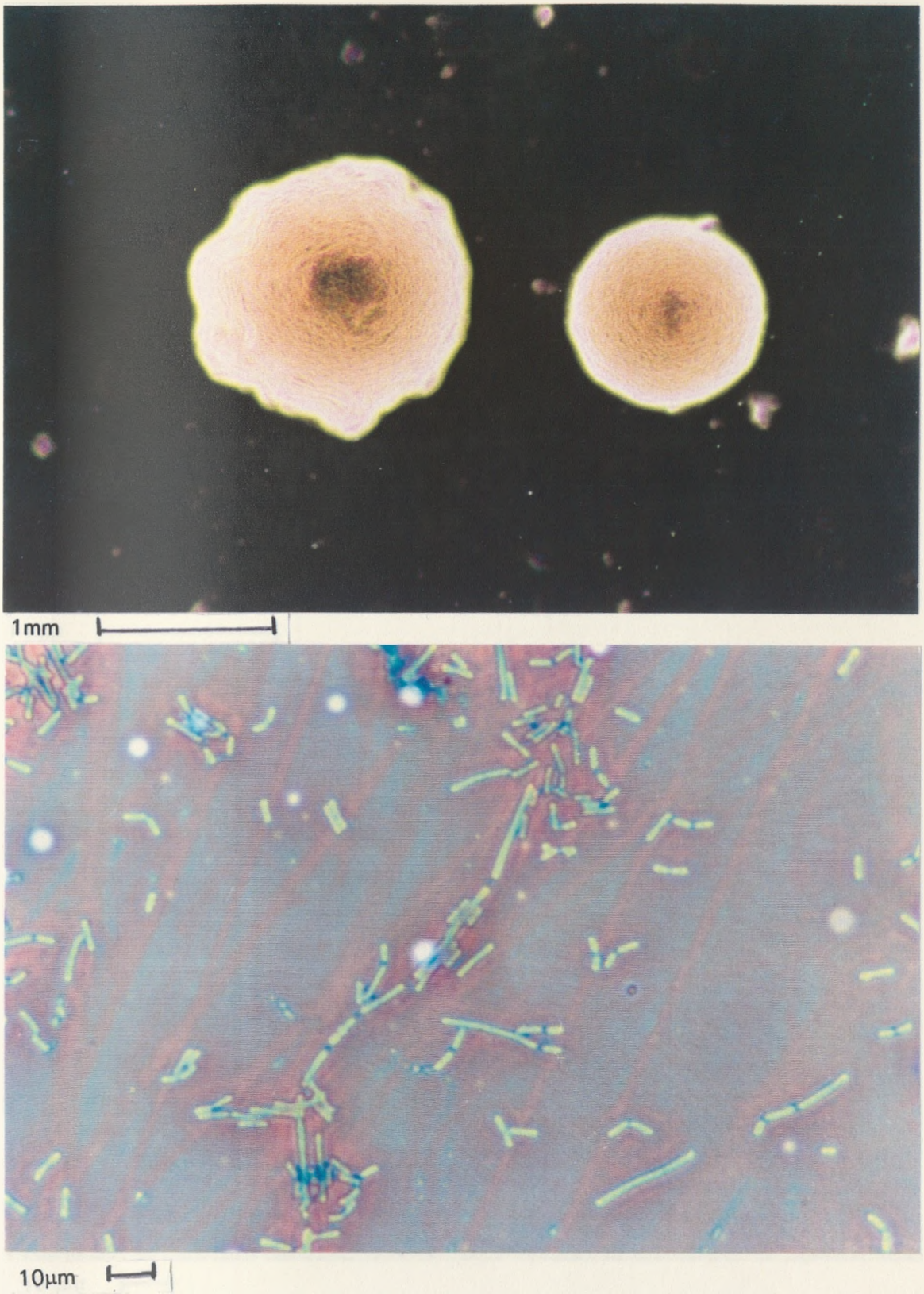
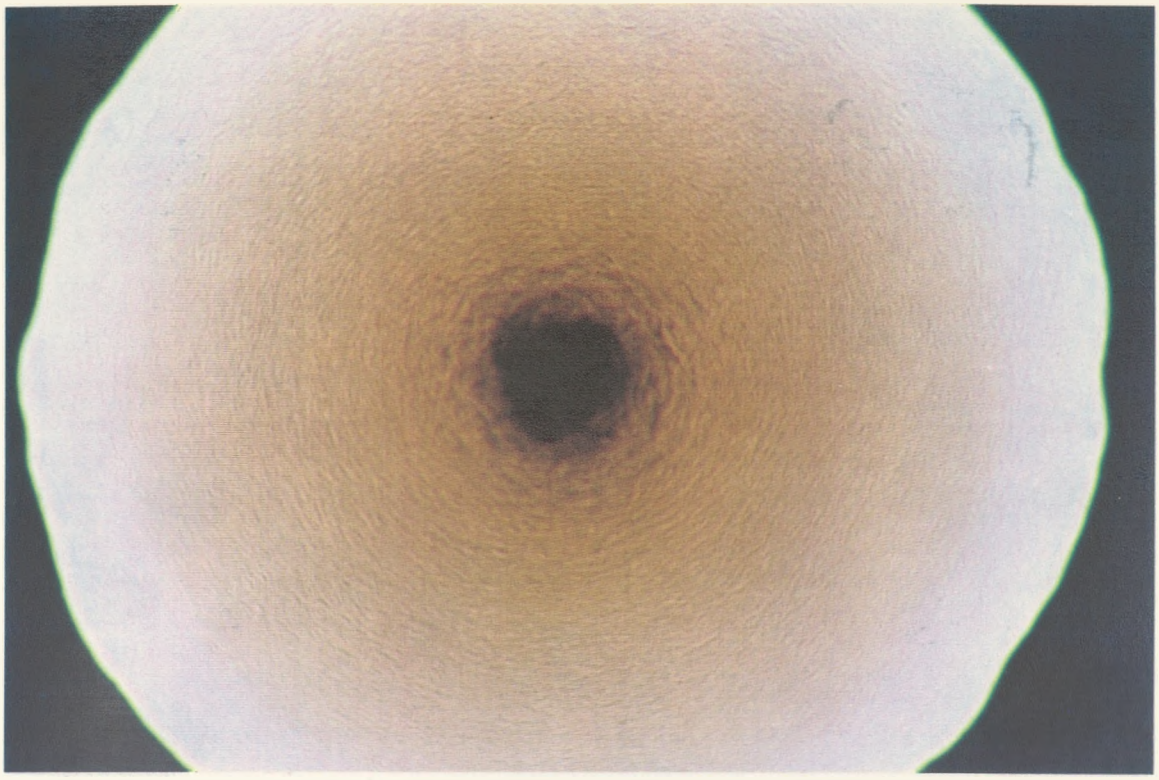


Figure 4.3 Growth of rough form of *L. monocytogenes* (NRB2) on TSYEA plates after 24 hours at 25°C.



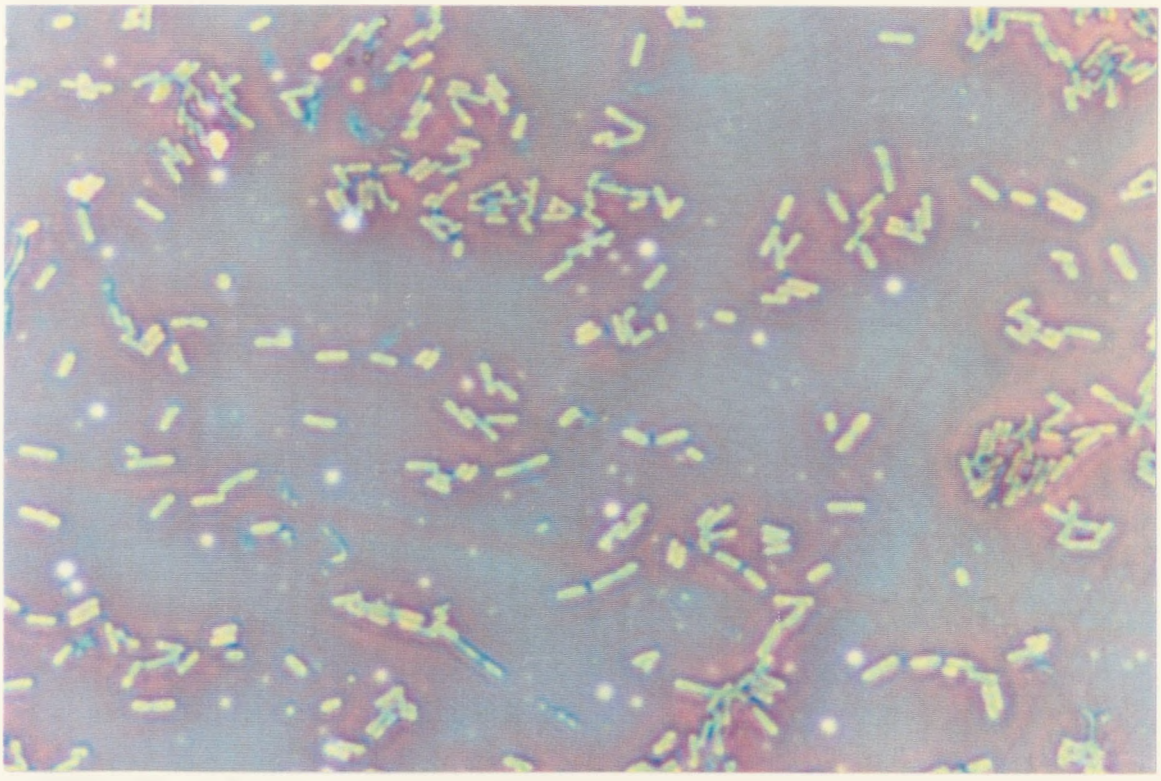
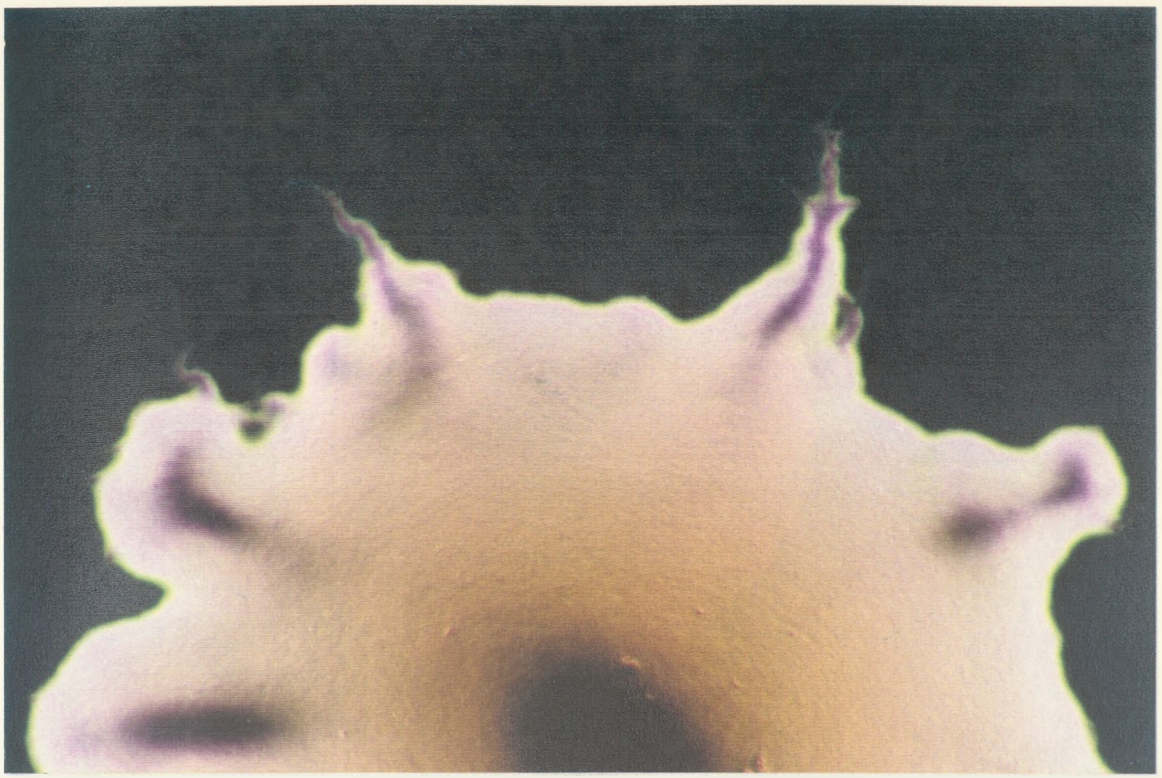


Figure 4.4 Growth of smooth form of *L. monocytogenes* (NCTC 11994) on TSYEA plates after 48 hours at 25°C.



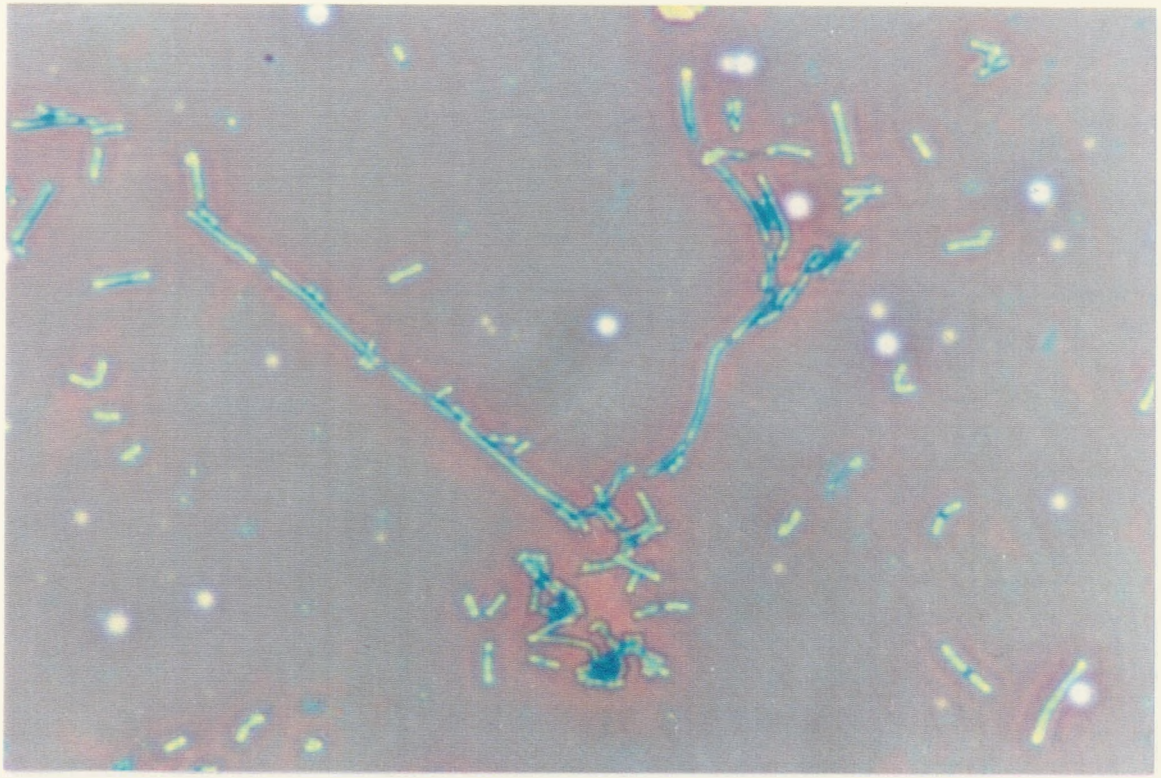


Figure 4.5 Growth of rough form of *L. monocytogenes* (NRB2) on TSYEA plates after 48 hours at 25°C.

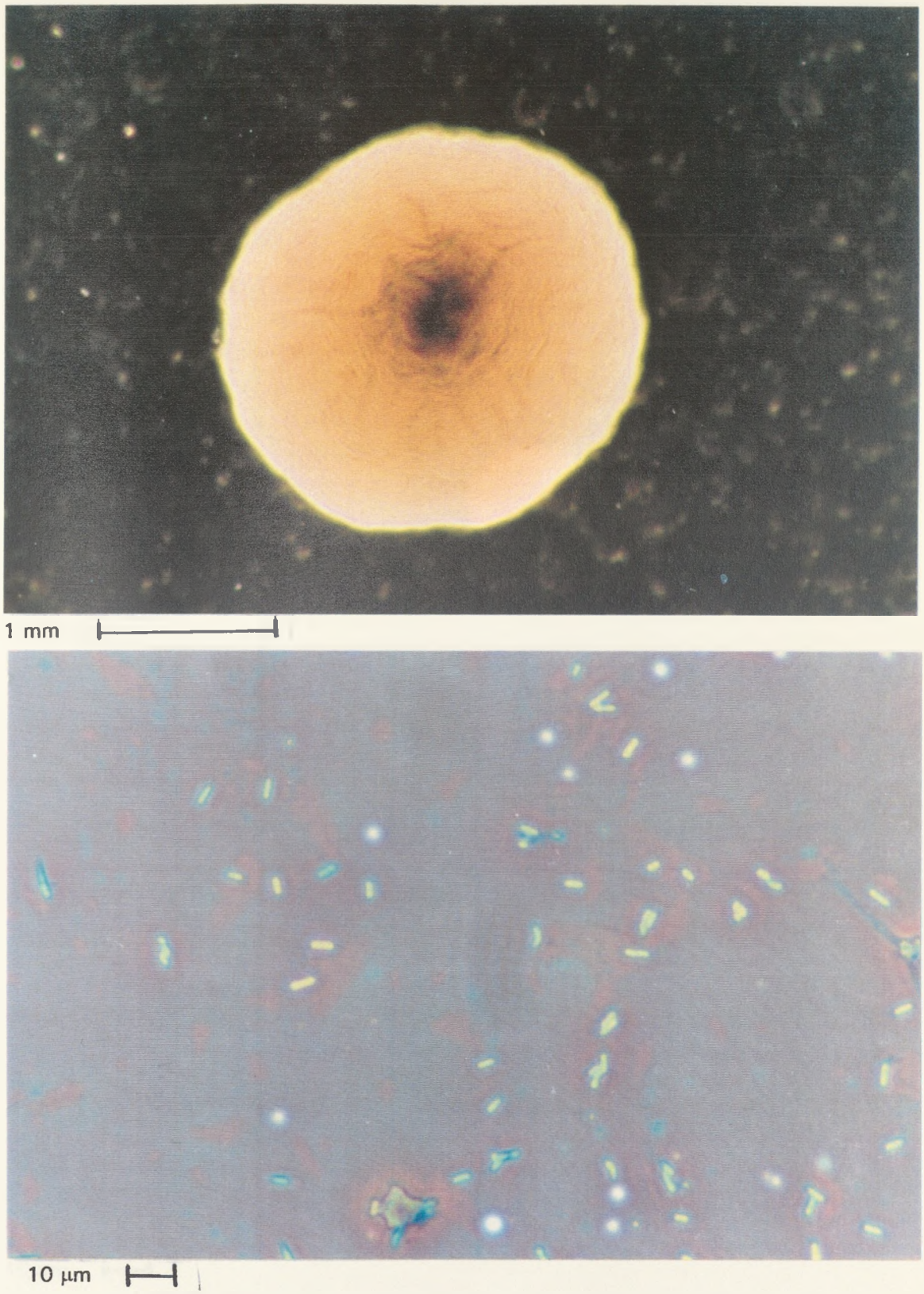


Figure 4.6 Growth of smooth form of *L. monocytogenes* (NCTC 11994) on LSA plates after 24 hours at 25°C.

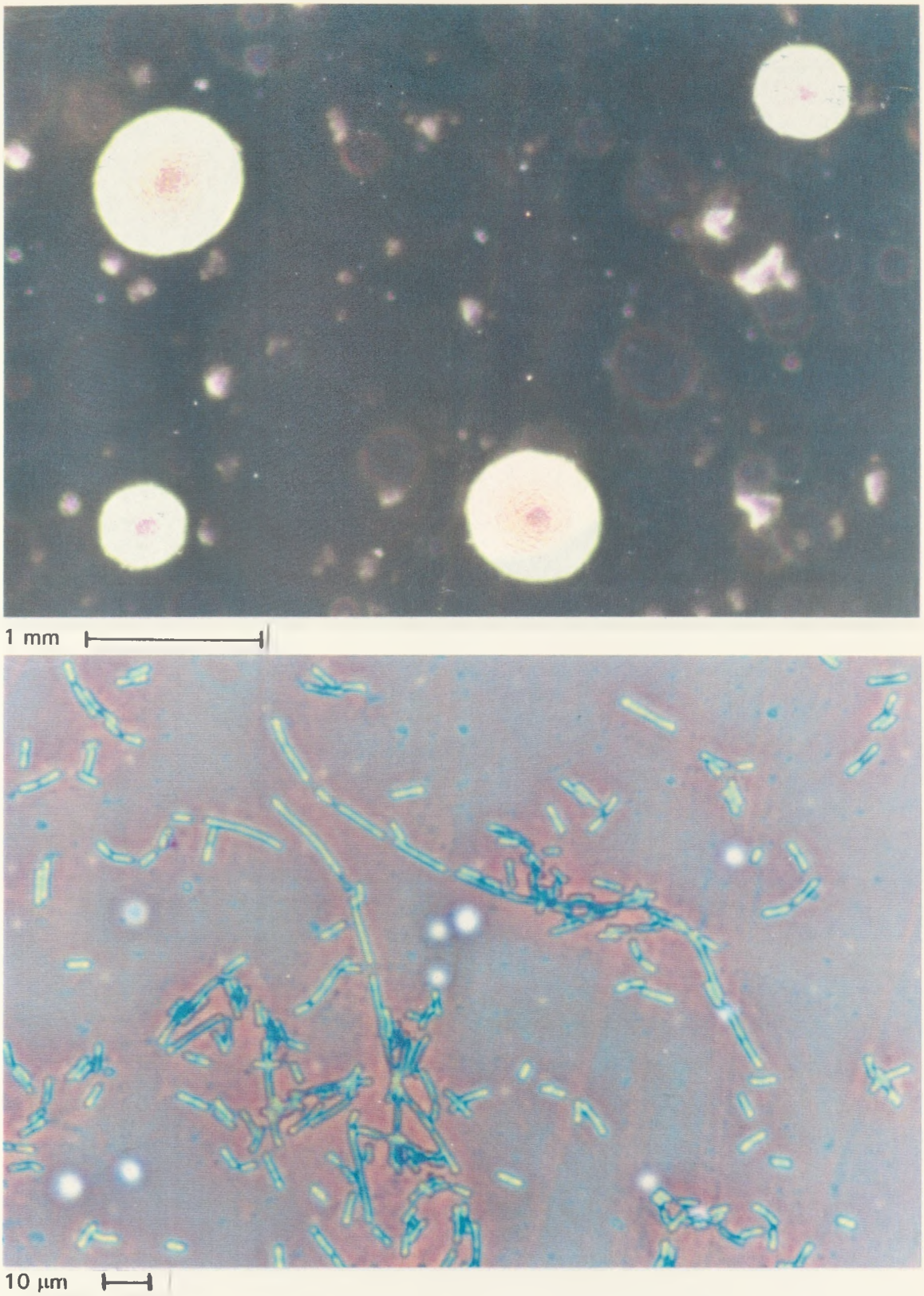


Figure 4.7 Growth of rough form of *L. monocytogenes* (NRB2) on LSA plates after 24 hours at 25°C.

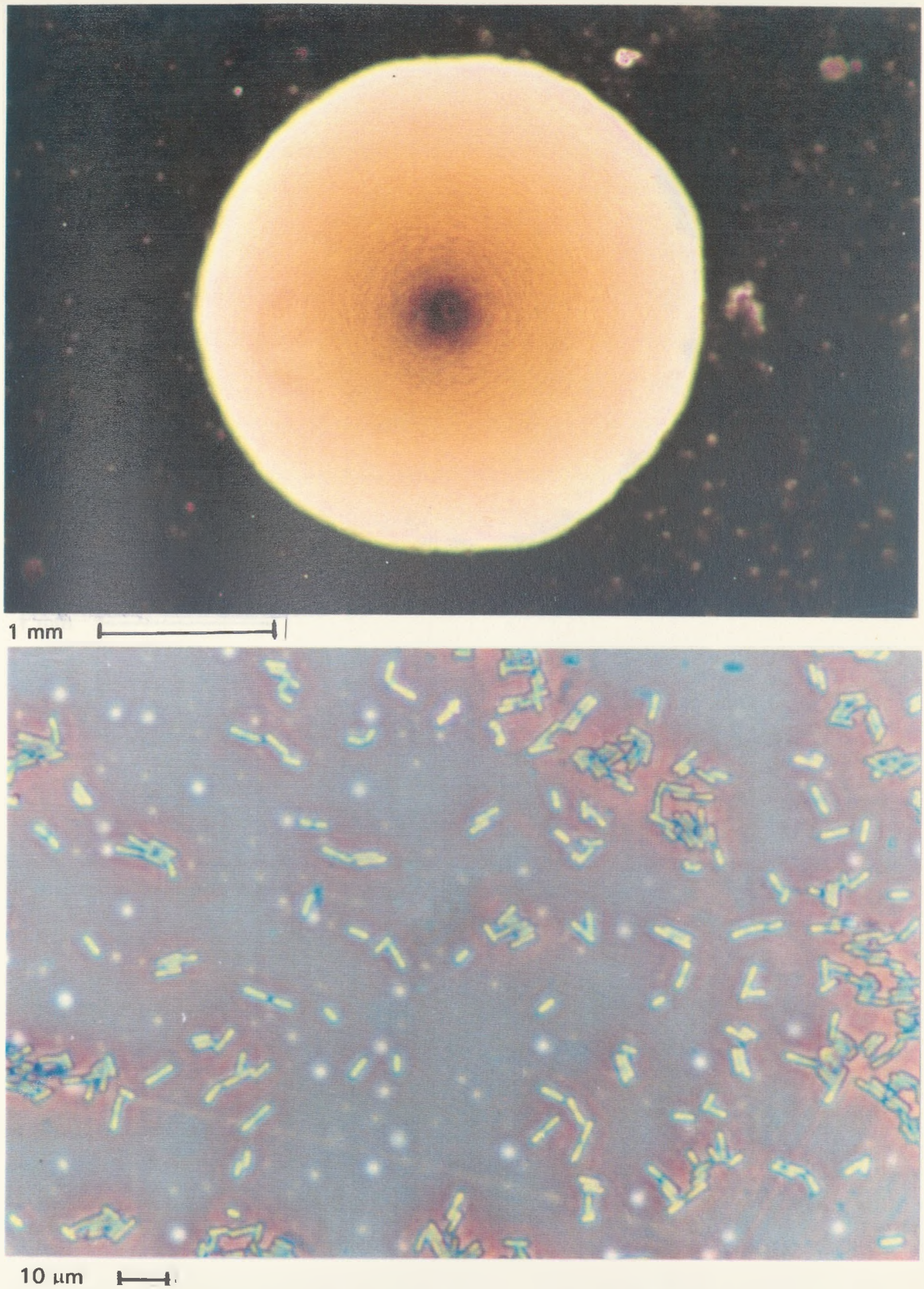
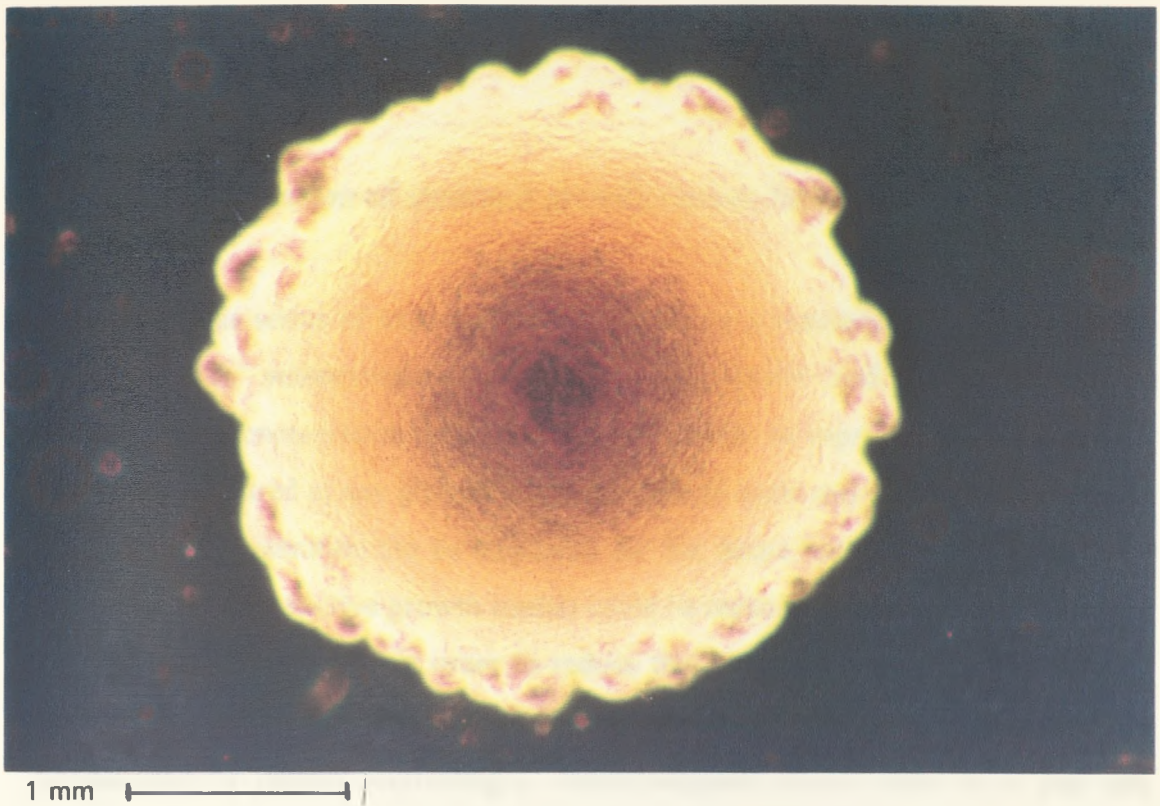


Figure 4.8 Growth of smooth form of *L. monocytogenes* (NCTC 11994) on LSA plates after 48 hours at 25°C.



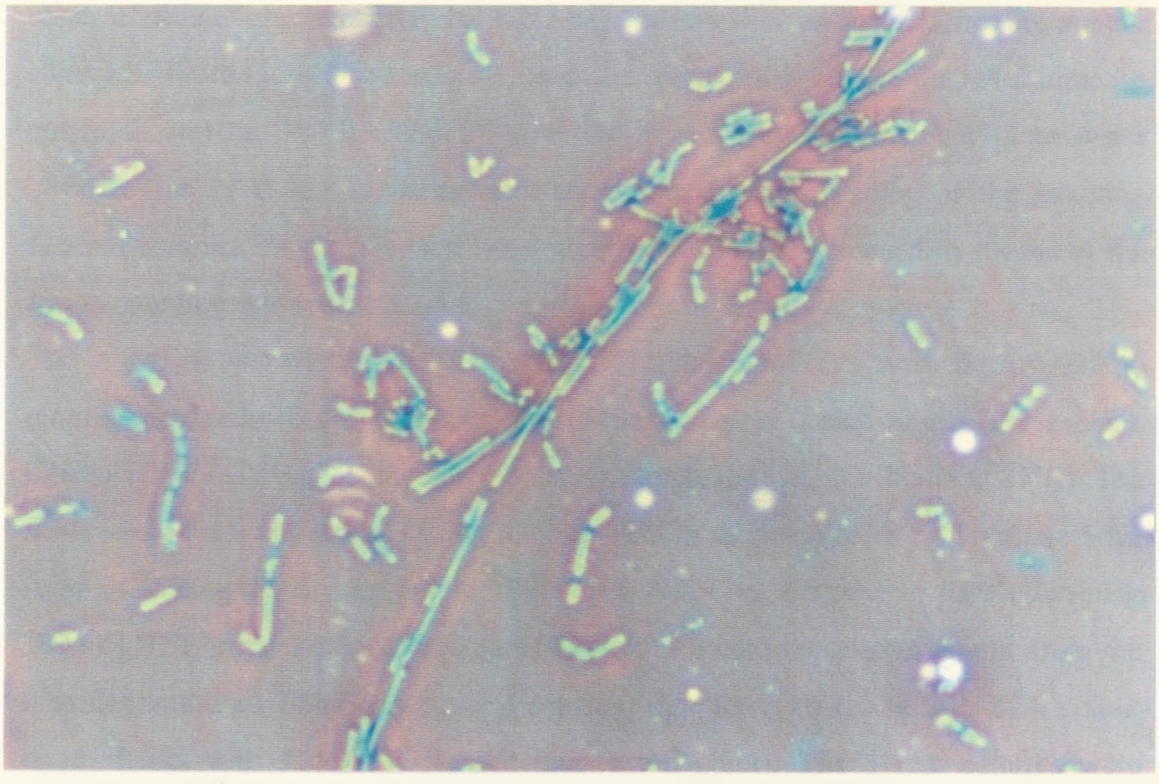


Figure 4.9 Growth of rough form of *L. monocytogenes* (NRB2) on LSA plates after 48 hours at 25°C.

(FDA) and United States Department of Agriculture (USDA) to recover these damaged cells could be improved by the implementation of these optimised culture conditions.

4.1.2.1 Construction of standard growth curves for *L. monocytogenes.*

The aim of this series of experiments was to obtain a standard growth curve for 3 strains of *L. monocytogenes* (NCTC's 9863, 11994 and 10357) so that a homogeneous concentration of cells (CFU ml^{-1}) could be reproducibly obtained and accurately measured at any given point in the growth cycle and a starting inoculum of known cell concentration (to be employed for the future inoculation of a variety of selective and non-selective growth media) could be reproducibly obtained.

The spiral plater was used in order to reduce the amount of time, consumables and labour involved in determining these growth cycles (section 3.5.6). It was standardised by initially cultivating *L. monocytogenes* NCTC 9863 to a final cell concentration of approximately 10^8 CFU ml^{-1}, serially diluting this spectrophotometric adjusted culture (at 440nm) before spread and spirally plating each diluted sample (in quadruplicate) on TSYEA and LSA plating media. Both sets of agar plates were incubated at 37°C for 48 hours prior to enumeration. Counting of the resulting *Listeria* colonies on plates which were spirally diluted was achieved by means of the 10 cm counting grid (section 3.5.6).

The stages of growth for the 3 strains of *L. monocytogenes* were determined by plotting either the O.D. measurements (440nm) and/or Total Aerobic Mesophilic Counts (CFU ml^{-1}) against the corresponding sample time period (hours at 37°C). The study was initiated by streak plating the organism to single colonies on Tryptone Soya Agar containing 0.6% yeast extract (TSYEA). A 250 ml Erlenmeyer flask containing 100 mls BHI broth was loop inoculated in duplicate with an isolated colony from the overnight cultured TSYEA plate at 4 separate time intervals (i.e. a total of 8 colour coded flasks with 1 control at each inoculation point). The 3 test cultures were inoculated at 4 separate time intervals (1000 hrs, 1400 hrs, 1800 hrs and 2000 hrs)

into the respective BHI broths in order to obtain samples from each stage in the growth cycle (i.e. from the early log growth phase to the stationary phase).

The test cultures were incubated at 37°C under aerobic conditions (110 rpm) over a 32 hour sample period (section 3.5.19). Duplicate 4 ml samples from each test culture was aseptically removed every 2-3 hours and subsequently analysed for cell concentration (via a turbidity measurement at 440nm and a Total Aerobic Mesophilic Count, CFU ml^{-1}). In order to ease the workload, two sets of experiments were carried out in duplicate, the first determined the growth cycle for strain NCTC 9863 and the second set established the stages of growth for strains NCTC 11994 and 10357.

The Total Aerobic Mesophilic Count (CFU ml^{-1}) was achieved by making decimal dilutions of each duplicate time sample (i.e. transferring 1 ml the respective test culture into 9 ml 0.01 M PBS) and spread plating the 0.1 ml aliquots (from these non-diluted and diluted samples) onto triplicate TSYEA and *Listeria* Selective Agar (LSA, Oxford formulation) plates. All these seeded agar plates were incubated at 37°C for 24 and 48 hours prior to enumeration. Corresponding turbidity measurements (λ_{440nm}-Abs/units) were made by aseptically transferring duplicate 1 ml samples into sterile 1.5 ml Eppendorf tubes, where the test cultures were subsequently centrifuged at 11,500 x g for 10 mins at 4°C (section 3.7.1). The sample was washed (x3); resuspended in 1 ml 0.01M PBS; transferred to a 1 cm light path cuvette and then measured using the spectrophotometer at 440 nm (section 3.6.1). The spectrophotometer was zeroed in air and the 0.01M PBS sterile blank was measured along with the resuspended samples (the PBS blank measurement was then subtracted from each sample absorbancy measurement).

Plate controls were made of the work environment (air), the equipment (e.g. sterile plastic spreaders, tips etc.) and culture media (BHI broth, TSYEA and LSA plating media). All these control plates were incubated along with the seeded culture plated at 37°C for 24 and 48 hours.

The mean Total Aerobic Mesophilic Counts (n= 20 measurements) for a 24 hour culture of *L. monocytogenes* (using the 10^{-4} dilution) was revealed to be $2.13x10^{4}$ CFU ml^{-1} for samples serially diluted and spread plated and $2.2x10^{4}$ CFU ml^{-1} for the

same samples which had been spirally plated on TSYEA plates. Both methods did not significantly differ at the P 0.05 level and good correlation was obtained (r=0.98). However, the spiral plating technique cannot be used for diluting and plating samples which contain a *Listeria* cell concentration $\geq 4.0 \times 10^5$ CFU ml^{-1} , as this was revealed to be the upper limit for accurate cell measurement in a non-diluted sample (i.e. the maximum number of *Listeria* colonies countable in the outer sector 3c of a 10 cm agar plate was 100). Therefore, if the test culture contains more than this upper limit of detection, then dilution of the sample to within the range of 10^3 to 10^5 CFU ml^{-1} would be required prior to further spiral plating.

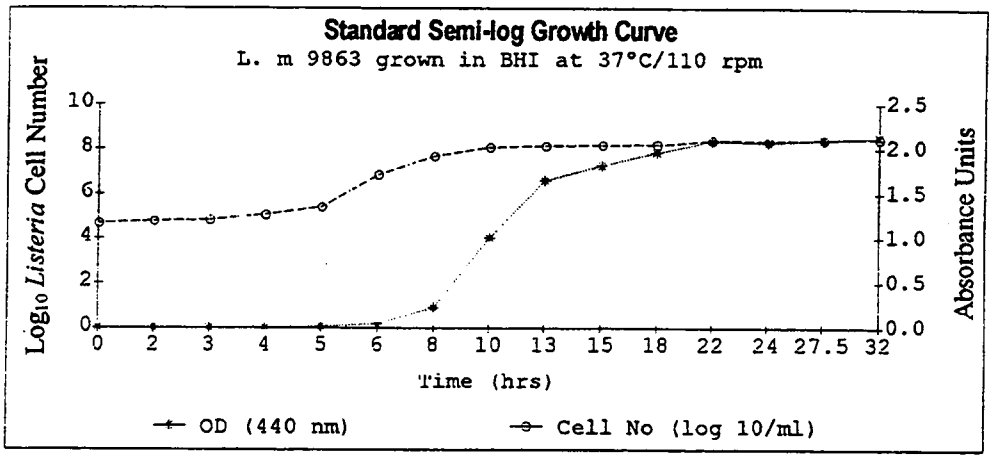


Figure 4.10 Determination of the phases of growth for *L. monocytogenes* (NCTC 9863) by way of turbidity measurements ($\lambda_{440\ nm}$) and corresponding Total Aerobic Mesophilic Counts (CFU ml^{-1}) on TSYEA plates over a 32 hour cultivation period (37°C at 110rpm).

The stages of growth for *L. monocytogenes* (NCTC 9863) were determined by plotting the results the log cell number (Total Aerobic Mesophilic Counts CFU ml^{-1}) and corresponding O.D. measurements at 440nm (appearing on opposite y-axis), against the sample time intervals (shown on the x-axis, hours) (Figure 4.10).

Figure 4.10 confirms that the absorbance measurements (Abs/units) carried out at the λ_{440nm} can be successfully employed to indirectly estimate the concentration of *Listeria* cells (log_{10} CFU ml^{-1}) at any given time interval under the present set of experimental conditions. Indeed, the minimum threshold concentration (CFU ml^{-1}) for which the spectrophotometer can be used to detect suspended *Listeria* cells at a wavelength of 440 nm was demonstrated to be approximately $5.0x10^4$ CFU ml^{-1}. Therefore, a microbiological load (i.e. for *L. monocytogenes* NCTC 9863) of less than this critical turbidity value will not be detected.

The results (cell concentrations with the corresponding turbidity values) from each series of colour coded cultivation broths were collated together and averaged to construct the standard growth curves shown in Figure 4.10. The starting inoculum for each freshly inoculated series of BHI test cultures varied in suspended cell concentration (CFU ml^{-1}), this was possibly due to the uneven concentration of cells on the inoculating loop or rod, consequently the initial inoculum in each culture flask was not uniform. Furthermore, it is well recognised that using a section of a colony to seed a set of sterile broths (i.e. with the specific intention of attaining uniformity in cell number and physical state in each complimentary flask) is not good microbiological practice, as no two samples taken from the same isolated colony are the same.

As this method of broth inoculation does not provide a uniform starting inoculum of 10 to 100 cells ml^{-1} for each culture medium, cells should be cultivated to a final O.D. measurement (λ_{440nm}) of approximately 2.0 Abs units (which corresponds to a cell concentration of ~ 10^8 CFU ml^{-1}), serially diluted to give a suspended cell concentration of approximately 10^4 cells ml^{-1}, and finally 1 ml of this dilution should be aseptically inoculated into 100 mls of fresh media to get the required level of 10 to 100 *Listeria* cells ml^{-1}.

A similar growth curve (absorbance measurements $_{440nm}$ plotted against cell concentration CFU ml^{-1}) was exhibited by all three strains of *L. monocytogenes* (Figure 4.11). These standard growth cycles will be employed to determine the *Listeria* cell concentration in future studies.

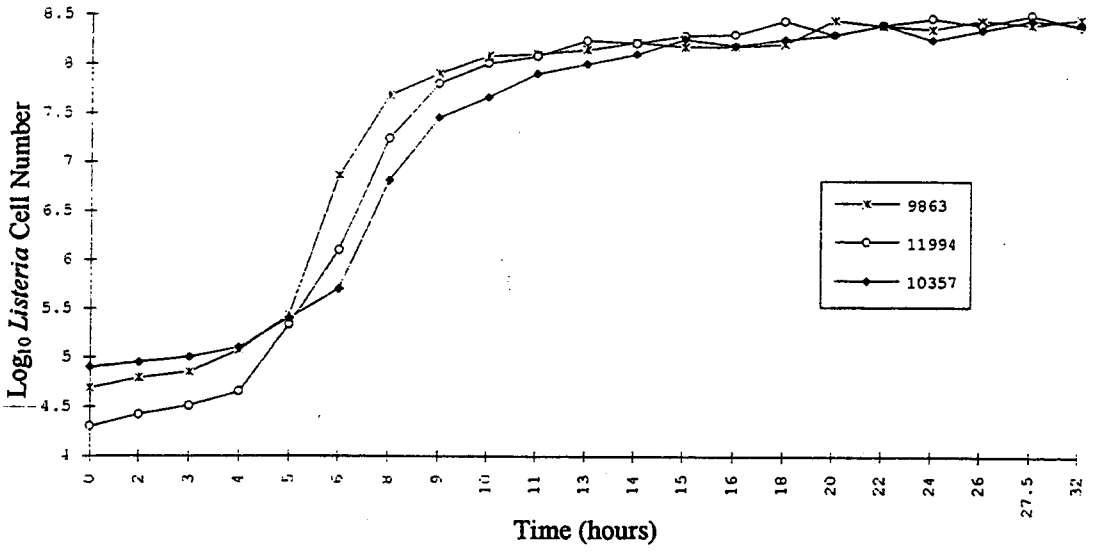


Figure 4.11 Standard growth curves for *L. monocytogenes* (NCTC 10357, 11994 & 10357) cultivated over a 32 hour period at 37°C (110 rpm).

Using the information presented in Figure 4.11, a starting inoculum of known cell concentration should be achieved in fresh sterile growth media by way of turbidity measurements (which should permit an immediate approximation as to the sample cell concentration without having to wait 24 hours for the sample to grow on an agar plate). Under the present set of controlled conditions a reproducible, known concentration of cells can be generated in a given broth at any desired cell concentration (CFU ml^{-1}).

4.1.2.2. Studies on the thermal resistance and recovery of heat damaged *Listeria* cells in laboratory based media and infant milk formulae

During the course of this research a series of experiments were carried out to establish whether or not the provision of certain parameters known to govern microbial heat resistance would enhance the thermotolerance of this organism. This information was then employed to determine whether these optimised culture conditions would allow

the recovery of low numbers of uninjured and sublethally injured *Listeria* cells which may be present in commercially available infant milk formulae. Factors shown to influence the emergence of pleomorphic cell and colony forms of *Listeria* in reconstituted infant milk formulae (IMF) were identified. The heat resistance of different strains and morphological forms was analysed under these optimised culture conditions in both laboratory based media and reconstituted infant milk formulae. The section was concluded by identifying suitable non-selective and selective resuscitation broths which improved the recovery of heat treated *Listeria* cells in reconstituted infant milk formulae; and established whether or not the recommended *Listeria* enrichment techniques (e.g. FDA and USDA methods) provides the same level of recovery.

4.1.2.2.1 Determination of the heat resistance characteristics of *L. monocytogenes* via end point recovery at 62.8°C.

This is the first in a series of experiments which focuses on the establishment of a defined set of growth, heating and recovery conditions that will optimise the survival and/or recovery of heat stressed cells and will allow the heat resistance of *L. monocytogenes* to be defined and modified. In the previous section, three standard growth curves for different strains of *L. monocytogenes* were obtained, the information supplied in each standard curve served to generate and immediately estimate a fixed cell concentration via turbidity measurements $_{440\ nm}$.

In order to identify the optimum recovery conditions for cell survival, a standard heating procedure was established where a known cell culture (~10^8 cells ml^{-1}) was heat treated and their recovery monitored. Indeed, this initial thermal study was designed to establish whether or not *Listeria* cells could tolerate the bactericidal temperatures employed in the holder type pasteurisation process (i.e. 62.8°C for 30 mins holding). This initial experiment laid down the foundations for future thermal inactivation studies, where improvements (seen as beneficial modifications in the experimental design) could be made in order to enhance the recovery of heat stressed (uninjured and injured) *Listeria* cells.

The study was initiated by generating a fixed homogeneous cell concentration of *L monocytogenes* (NCTC 8963), which is an essential requirement for identifying subtle or significance differences in thermal resistance capabilities between strains and/or culture conditions. This was achieved by initially streaking to single colonies on TSYEA plates (i.e. plates were incubated at 37°C for 24 hours). An isolated colony was then loop inoculated into duplicate 250 ml Erlenmeyer flasks which contained 50 mls of BHI broth (subculture medium). This subculture medium was incubated at 110 rpm for 24 hours at 37°C where the cell density was spectrophotometrically adjusted (λ_{440nm}) to a turbidity measurement of approximately 2.2 Absorbance units with 0.01 M PBS (i.e. adjusted at A_{440nm} to 2.2 then washed x3 in PBS and centrifuged at 11,500 x g for 5 mins at 4°C) which corresponded to a suspended cell concentration of $5.0x10^8$ CFU ml^{-1}.

Decimal serial dilutions of this A_{440nm} adjusted culture were made (1 ml of culture into 9 ml PBS) and a 1 ml aliquot of the 10^{-5} dilution was inoculated into 100 mls of sterile BHI broth to give an initial test culture of approximately 10 to 100 cells. The test culture was again incubated under aerobic conditions (110 rpm) for 24 hours at 37°C to obtain a homogeneous cell distribution, after which it was spectrophotometrically adjusted (A_{440nm}) to an absorbance of 2.2 as described above.

This final concentration of *Listeria* cells was confirmed by spread and spirally plating decimal dilutions made of this test culture onto TSYEA and LSA plates. These seeded plates were incubated for 24 and 48 hours at 37°C prior to enumeration. At least 3 colonies per plate with the highest dilution of countable colonies were selected for confirmation.

Thermal inactivation of suspended *Listeria* cells was performed by initially pre - heating a waterbath (Techne Tempette Junior TE-8J) to a temperature of 62.8°C. When the bath had reached temperature, 20 ml aliquots (in triplicate) of this spectrophotometrically adjusted (A440nm) test culture were aseptically transferred into a series of 28 ml sterile McCartney screw cap bottles (which had been pre-sterilised by autoclaving at 121°C for 15 mins). The bottles were carefully selected to ensure that the structural integrity of each bottle was satisfactory (i.e. free of cracks and abrasions) and that the rubber seal fitted properly. To reduce the chances of

heated water ingress or infiltration, the interface between bottle and cap was wrapped with 3 layers of parafilm wax, thus sealing the bottle.

The bottles were secured to a wire basket with a strand of flexible wire and totally submerged at least 5 cm below the water level for the 30 mins holding period. A sterile heating menstruum control was also heated over the course of the 30 mins heating cycle. No allowance was made for the heating up period in the submerged heating menstruum prior to holding at 62.8 ± 0.2°C for 30 mins. The temperature of the waterbath and the heating menstruum (i.e. temperature of a control sample bottle which contained an equivalent volume of sterile BHI broth) was constantly monitored throughout the heat cycle by means of an inserted thermometer (Figure 4.12).

The heating menstrua (in addition to controls) were removed from the waterbath after the 30 mins heating and stored on ice. A triplicate trial was conducted for the same strain of *L. monocytogenes* (i.e. 3x2 test cultures). Viable counts of surviving populations from the heat treated test culture (in addition to a non-heated test culture control) were immediately determined on TSYEA and LSA (with and without additional enrichment) by means of serial decimal dilutions and spiral plating. The seeded plating medium containing the heat treated *Listeria* cells were incubated for 24 and 48 hours at 37°C.

Duplicate 5 ml aliquots from the heat treated heating menstruum were aseptically transferred into a series of 500 ml Erlenmeyer flasks each containing 50 ml of the following enrichment medium, where they were orbitally incubated at 110 rpm for up to 7 days at 37°C:

1. Tryptone soya broth containing 0.6% yeast extract (TSYEB)
2. Brain heart infusion broth (BHI)
3. *Listeria* enrichment broth (formulated by the Food and Drug Federation) (LEB-FDA)
4. *Listeria* enrichment broth containing antibiotic supplement(formulated by the Food and Drink Federation) (LEBs-FDA)
5. *Listeria* enrichment broth (formulated by the U.S Department of Agriculture) (LEB-USDA)
6. *Listeria* enrichment broth containing antibiotic supplements (formulated by the U.S. Department of Agriculture) (LEBs-USDA)
7. Nutrient broth number 2 (NB-2)
8. Nutrient broth (NB)

9. Nutrient broth containing 0.25% glucose (NGB)
10. Nutrient broth containing 0.25% glucose and stored under static conditions (NGBst.)
11. BHI non-heat treated test culture control
12. BHI sterile medium control

Samples were taken after 0 hour, 20 hour, 40 hour and 7 days storage (as described earlier) where a turbidity measurement (A_{440nm}) and total aerobic mesophilic count (CFU ml^{-1}) on TSYEA and LSA were carried out. The seeded plates were incubated for 48 hours at 37°C.

The Total Aerobic Mesophilic Count taken from the non-heat treated test culture control revealed that $7.9x10^8$ *L. monocytogenes* (NCTC 9863) cells ml^{-1} were heated in a BHI broth suspension at 62.8 ± 0.2°C for 30. No allowance was made for the time required for the heating menstruum to reach the actual holding/heating period of 62.8°C (i.e. no heating up period observed), therefore the total time at which the cells experienced a bactericidal temperature of 62.8°C was in fact less than the required 30 mins.

Immediately after this heat exposure, a measure of the reduction in *Listeria* cell number or thermal inactivation was achieved by plating a sample of the test culture/heating menstruum onto TSYEA and LSA plating media. The number of sublethally injured cells being determined as the difference in colony counts between the non-selective (TSYEA) and selective (LSA) plating media. However, surviving colonies did not develop on either of the plating media after 48 hours incubation at 37°C. Therefore, a 30 minute exposure at 62.8°C ± 0.2°C reduced the test culture from $7.9x10^8$ CFU ml^{-1} to <1 CFU ml^{-1} (i.e. pour plating of 1ml of the neat dilution) on both plating media. This indicates the the present combination of cultivation, heat treatment and recovery conditions was inadequate for the purpose of monitoring thermal resistance.

A seven day enrichment of these heat treated *Listeria* cells did not result in recovery of *L. monocytogenes* on either of the plating media (Table 4.5). Indeed over the course of this storage period the turbidity measurements did not increase from their base line value of ~ 0.2 absorbance units, which was reflected in the failure to recover any heat damaged cells on the various plating media (ND at 10^2 CFU ml^{-1}). Each of

the various enrichment control broths inoculated with 5 ml of a non heat treated test culture control of *L. monocytogenes* supported growth, with an initial mean cell concentration of 3.3×10^7 CFU ml^{-1} for the 0 hour stored samples.

Figure 4.12 Position of submerged thermal inactivation bottle during heating in waterbath.

As no *Listeria* colonies were identified due to the harsh thermal conditions, no visual search for pleomorphic surface variants could be performed. In order to determine the potential heat resistance of *L. monocytogenes* (under a particular set of experimental conditions) and/or to investigate certain parameters which may protect the cells (or prepare them for the sudden exposure to bactericidal temperatures) certain modifications to the heating regime were made.

Table 4.5 illustrates the inability of various non selective and selective enrichment broths to recover heat treated cells of *L. monocytogenes* (NCTC 9863)

Enrichment medium†	Absorbance at 440 nm (Abs/units)				Total Aerobic Count (CFU ml^{-1})			
	0 hr	20 hr	40 hr	7 d	0 hr	20 hr	40 hr	7 d
BHI	0.255	0.201	0.216	0.188	ND‡	ND‡	ND‡	ND‡
TSYEB	0.258	0.205	0.211	0.193	ND‡	ND‡	ND‡	ND‡
LEB-FDA	0.259	0.211	0.221	0.188	ND‡	ND‡	ND‡	ND‡
LEBs-FDA	0.262	0.211	0.215	0.173	ND‡	ND‡	ND‡	ND‡
LEB-USDA	0.210	0.154	0.173	0.144	ND‡	ND‡	ND‡	ND‡
LEBs-USDA	0.214	0.163	0.177	0.155	ND‡	ND‡	ND‡	ND‡
NH-2	0.252	0.202	0.215	0.185	ND‡	ND‡	ND‡	ND‡
NB	0.255	0.206	0.211	0.195	ND‡	ND‡	ND‡	ND‡
NGB	0.261	0.205	0.211	0.200	ND‡	ND‡	ND‡	ND‡
NGBst.	0.256	0.213	0.214	0.201	ND‡	ND‡	ND‡	ND‡

† refer to the experimental protocol for a definition of the enrichment broth abbreviations.
‡ND refers to no cells detected at the lowest dilution sampled (i.e. 10^{-1})

Using the same thermal inactivation procedure, *L. monocytogenes* (NCTC 9863) was heat treated over shorter exposure times (i.e. 0, 3, 6, 9, ...30 mins at 62.8 ± 0.2°C) and the thermally treated test cultures were spread plated directly onto TSYEA and LSA plates (without being enriched). While a heating up period of 5 mins 52 seconds was determined and allowed for when calculating the total exposure time at 62.8°C (monitoring the rise in temperature using an inserted thermometer, Figure 4.13), no samples were removed from this period for microbiological analysis.

Despite introducing shorter exposure times at 62.8 ± 0.2°C, no *Listeria* colonies appeared on either plating media over a 72 hour incubation period (even at 10^{-5}, the lowest dilution sampled at the point where the temperature of the test culture reached 62.8°C, i.e. 0 mins holding).

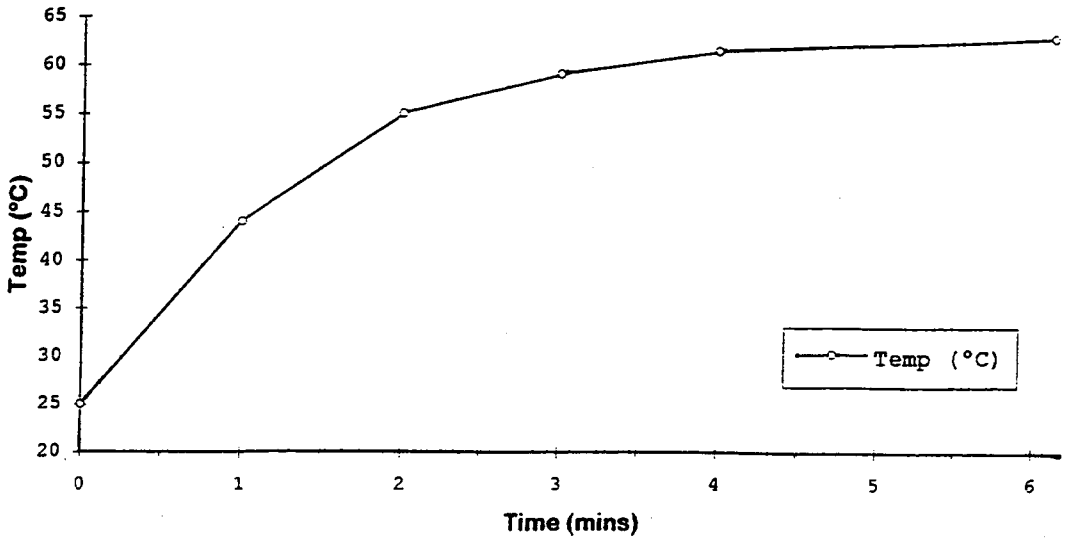


Figure 4.13 Rate of temperature increase in the BHI broth heating menstruum during the heating up time period to 62.8°C.

The same thermal inactivation procedure was repeated making allowances for the bactericidal effects of the warming up period (in this case the test culture took 6 mins 8 seconds to reach the holding temperature of 62.8 ± 0.2°C). Samples from the warming up period were taken every 1 min for microbiological analysis as described in section 4.2.2.1. However, as there was no guarantee that either uninjured and/or sublethally damaged *Listeria* cells would be recovered during this thermal inactivation process, samples were simply spread plated onto TSYEA and LSA plates immediately after heat treatment (without enrichment) and incubated at 37°C for 24 and 3 days.

A plot of the $\log_{10}$ number of cell survivors against the corresponding heat inactivation temperature (experienced during warming up period), revealed that the bactericidal effect of the warming up period reduced the *Listeria* cell population from 10^9 CFU ml^{-1} to $1.2x10^1$ CFU ml^{-1} and <1 CFU ml^{-1} for samples enumerated after 24 hours incubation on TSYEA and LSA plates respectively (Figures 4.14 and 4.15).

As the non-selective plating medium (TSYEA) supported the growth of more *Listeria* colonies, it is proposed that the selective plating media (LSA) supported the growth of uninjured cells, while TSYEA recovered both uninjured and sublethally damaged cells.

Examination of these Figures 4.14 and 4.15 revealed that the duration of incubation (i.e. additional 48 hours) improved the recovery of heat treated cells on both plating media at each heat inactivation temperature. Therefore, the greatest recovery of *L. monocytogenes* cells occurred on TSYEA plates after 24 hours storage, while a further 48 hours incubation resulted in both plating media recovering a similar level of *Listeria* cells.

4.1.2.2.2 Application of a mild heat shock (tempering) to *L. monocytogenes* cells prior to heating to and holding at 62.8C in order to determine the effect on thermal resistance.

Under the presently defined thermal treatment conditions, an initial test culture of ~ $9x10^8$ CFU ml^{-1} will be reduced to $1.4x10^1$ CFU ml^{-1} (i.e. the total aerobic mesophilic count achieved at T0 mins on TSYEA plates stored over 24 hours at 37°C) by the time it takes for the temperature of the heating menstruum to equilibrate with a holding temperature of 62.8°C. Therefore, under these present experimental conditions, this initial cell concentration of ~10^9 cells ml^{-1} will be reduced to a non detectable level (<1 CFU ml^{-1}) after just 2 mins at 62.8C if the thermally stressed cells are recovered by the direct plating technique.

Although the cells of *L. monocytogenes* (NCTC 9863) were exhibiting weak thermal resistance at 62.8°C, the present experimental conditions (albeit quite severe on *Listeria* cell survival) can still suffice as a template where the application of controlled modifications to a few extrinsic parameters can be potentially monitored. Thus any increased thermal resistance achieved as a direct result of these modifications can be recorded and implemented at a later stage.

It has been documented that the extent of cell injury produced during sublethal heating is very much dependent on the following: time of exposure; physiological age of cells; cell density during heating; composition of the growth medium, injury

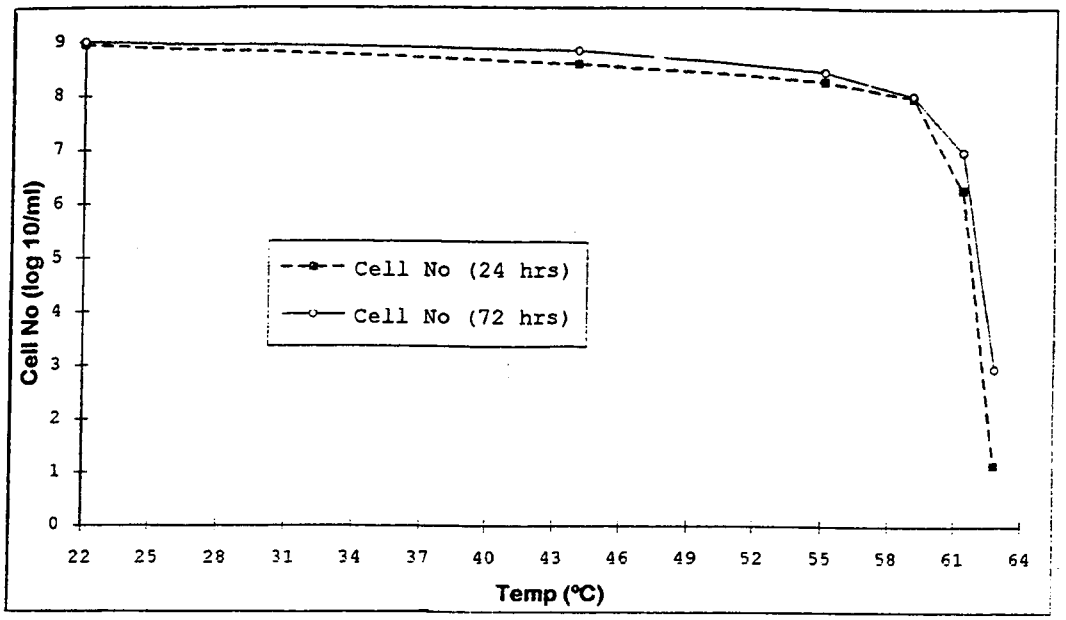


Figure 4.14 Recovery of heat treated *L. monocytogenes* (NCTC 9863) heated to and held at 62.8 ± 0.2°C, followed by enumeration on TSYEA plating medium over a 24 and 72 hour incubation period at 37°C.

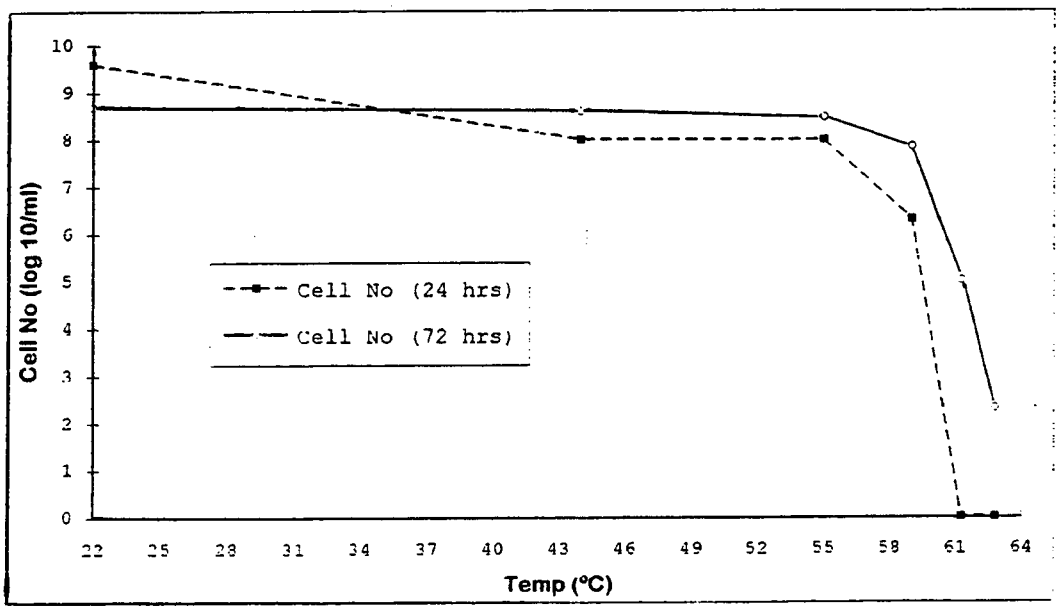


Figure 4.15 Recovery of heat treated *L. monocytogenes* (NCTC 9863) over a heating and holding period at 62.8 ± 0.2°C, followed by enumeration on LSA plating medium over a 24 and 72 hour incubation period at 37°C.

menstruum; temperature of exposure; temperature of cultivation prior to heat treatment; and heat shocking or tempering of cells prior to heat treatment. The provision of these conditions should theoretically influence the potential recoverability of thermally treated *Listeria* cells.

Previous reports (Fedio and Jackson 1989, Linton et al 1990) have demonstrated that the subjection of *L. monocytogenes* to a sublethal temperature for a brief time period prior to the actual full heat treatment can potentially enhance the thermal resistance capability of the treated cells. This brief exposure at temperatures which are considered to be a few degrees (centigrade) above the cells maximum growth potential, may possibly render the cells in a state of temporary stress whereby the organism achieves a temporary state of elevated thermal tolerance. This temporary state of heat shock may pre-condition the cells to the bactericidal effects of a sudden heat exposure.

This is the first in a series of experiments to incorporate a parameter (i.e. tempering) previously shown to influence the heat resistance of non-spore forming bacteria. The standard experimental procedure, as laid down in the previous thermal inactivation studies (section 2.1.2.2.1), was modified to include a heat shock stage (test culture elevated to a temperature of 47°C for 30 mins prior to subjection of the cells to pasteurisation at 62.8°C) with the hope that cells would tolerate longer exposure times at a heat inactivation temperature if they are initially held for a short period at above optimum growth temperatures.

As for the previous thermal inactivation studies, 20 ml aliquots of a *L. monocytogenes* test culture (again adjusted as A440nm to a cell concentration of ~10^9 CFU ml-1) were transferred to a series of 28 ml McCartney bottles and labelled as described in section (4.2.2.4). Two pre-heated waterbaths were employed, the first at a fixed temperature of 47°C and the second at the normal holding temperature of 62.8C. All the test culture/heating menstruua destined for thermal treatment at 62.8°C were placed in the former waterbath to temper at the sublethal heat shock temperature of 47°C for 30 mins. In achieving this tempering temperature of 47°C a heating up period of 1 mins 16 secs was allowed for. After this heat shock 30 min exposure, the test

cultures were cooled back to room temperature (22.3°C) prior to the main heat treatment at 62.8°C for 30 mins holding.

The test cultures were then placed in the main waterbath, heated to and held at 62.8°C for the various time intervals designated in the previous thermal study. Samples from the heat treated and the non-heat treated test cultures were spread plated onto TSYEA and LSA, where they were incubated at 37°C for 72 hours prior to enumeration. However, none of the samples were enriched to improve the recovery of sublethally damaged *Listeria* cells.

The results from Figure 4.16 revealed less cells (uninjured and/or injured) were recovered when a pre-conditioning tempering stage was introduced compared to directly heating and holding the cells at 62.8°C as shown in the previous thermal studies. When tempering was implemented in conjunction with a heat treatment at 62.8°C, the number of cells recovered by the direct plating technique at T0 mins was $1.5x10^1$ CFU ml^{-1} compared to $9.0x10^2$ CFU ml^{-1} (where the cells were heated immediately to 62.8°C without previous thermal pre-conditioning). These counts were recorded from TSYEA plates which were enumerated after 72 hours at 37°C. Therefore, it would appear that no improvement in the recovery of heat treated *Listeria* cells number was achieved by way of tempering under the present set of experimental conditions.

As no enrichment of the thermally treated test cultures occurred, it was possible that broth resuscitation might have improved the recovery of sublethally cells in both tempered and/or non-tempered test cultures of *L. monocytogenes*. It was also quite obvious that additional parameters would have to be incorporated into this recovery technique, as only a limited number of *Listeria* cells ($\sim10^2$ CFU ml^{-1}) was detected after 2 mins exposures at 62.8°C. The colony morphology did not vary throughout the experiment, irrespective of the plating media and/or heating conditions. Also the results indicated that the direct plating technique was a poor detection method for the recovery of heat treated *L. monocytogenes*.

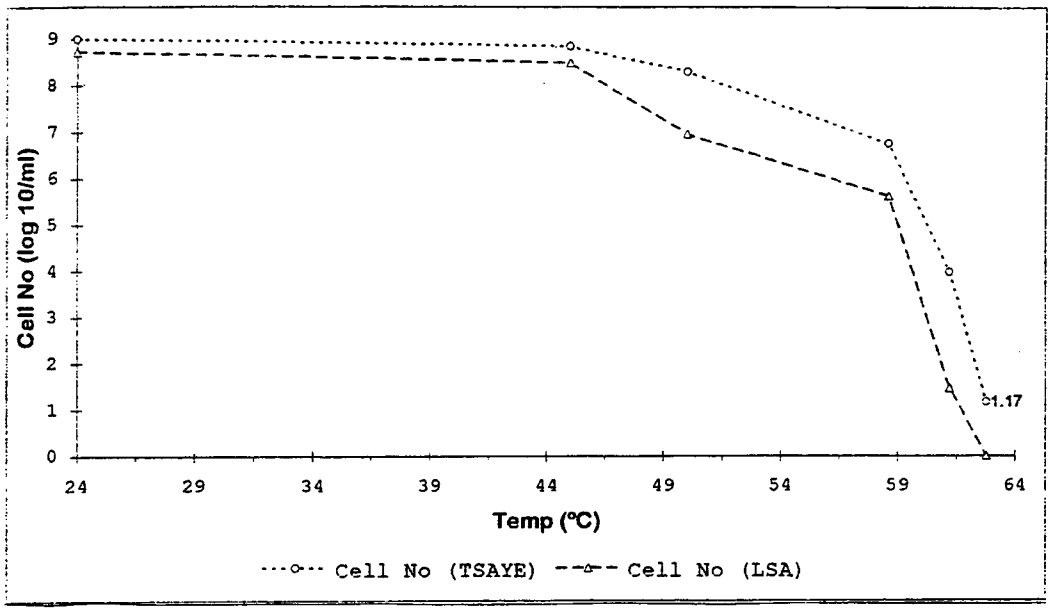


Figure 4.16 illustrates the thermal inactivation of tempered *L. monocytogenes* (NCTC 9863) cells during the heating up to a holding temperature of 62.8°C.

4.1.2.2.3 Confirmation that *Listeria* cells heat treated in previous thermal studies were in their stationary phase of growth.

Heat shocking of *Listeria* cells prior to thermal inactivation at 62.8°C did not appear to enhance the recovery of healthy and heat injured cells when enumerated by the direct plating technique without subsequent broth enrichment. It has been documented that microbial cells in stationary phase of growth are more tolerant to heat than cells in log phase (Linton *et al.*, 1990). This study was designed to establish whether cells heat treated in previous heating experiments were in their stationary growth phase. Indeed, in earlier non-thermal growth curves studies, an isolated colony from a 24 hour incubated TSYEA plate was directly inoculated into a BHI test culture broth and cultured at 37°C under aerobic conditions over a 32 hour period (section 4.1.2.1).

The resuspended *Listeria* cells experienced a relatively short lag period of 2.3 to 3 hours before onset of exponential growth. As the intention of future thermal studies was to heat treat cells which were considered to be homogeneous in both number and stage reached in growth cycle, it was important to construct a new growth curve for *L. monocytogenes* where cells in their mid -log to late log growth phase (i.e in a subculture medium) were inoculated into fresh test culture media of complimentary nutrient composition to that of the subculture medium.

Moreover, previous growth curves have demonstrated that a 24 hour *loop inoculated* test culture of *L. monocytogenes* had reached its stationary phase of growth prior to thermal treatment under the standard set of culture conditions (37°C at 110 rpm). However, loop inoculated test cultures frequently varied in cell concentration and this was probably due to loop being charged with a section of a colony (where the variation in cell concentration was significantly high). Subsequent thermal studies had been designed to specifically heat inactivate a 24 hour test culture of *L. monocytogenes* which had been *subcultured* at 37°C (110 rpm) for 24 hours prior to inoculation into the test culture medium.

The objective of this study was to establish whether or not this 24 hour test culture had achieved its stationary phase of growth and also to confirm that the *Listeria* cells removed from a 24 hour *loop* inoculated test culture had achieved a similar stationary growth phase. By cultivating each subculture medium under a standard set of conditions and then inoculating the test culture with a spectrophotometrically adjusted cell culture from a fixed time period, *Listeria* cells removed over the course of the growth cycle determination should have achieved equal growth phase status. For all future thermal inactivation studies, care was taken to ensure that the nutrient composition of the subculture medium was complimentary to that of the test culture which would help to avoid any unnecessary lag period.

A growth cycle was determined for the *Listeria* cells growing in a loop inoculated TSYE broth, BHI broth and full cream milk subculture medium (in this instance these subculture broth media are the test cultivation media) under aerobic conditions (110 rpm) for 24 hours at 37°C via turbidity measurements (A 440 nm) and total aerobic mesophilic counts (CFU ml^{-1}) over a 32 hour period as described previously.

As samples from the entire growth period were required (i.e. over the 0 hours to 32 hours), the test cultures were inoculated at 4 separate time intervals with a corresponding 10^{-5} dilution of an over night subculture broth. A sample from each test culture was taken every 2 to 3 hours for a turbidity measurement (A_{440nm}) and a total aerobic mesophilic count (CFU ml^{-1}) as described previously.

Results from this growth cycle study are illustrated in Figure 4.17, where *L. monocytogenes* NCTC 9863 had been cultivated in TSYEB, BHIB and full cream milk over a 30 hour observation period at 37°C. This figure confirmed that *Listeria* cells, subsequently transferred to other test broths (prior to heating) after 20 hours had reached the stationary phase of growth. Indeed, Figures 4.17 and 4.18 illustrated that the 20 hour test culture represents cells in their stationary period, irrespective of whether or not the test culture was initially loop inoculated or taken from a homogenous broth subculture. Although the test cultures exhibited a short lag period when they were initially inoculated with a loopful of *Listeria* cells, by the time these cells were 20 to 24 hour old they had reached either the late exponential or early stationary phase of growth (Figure 4.17).

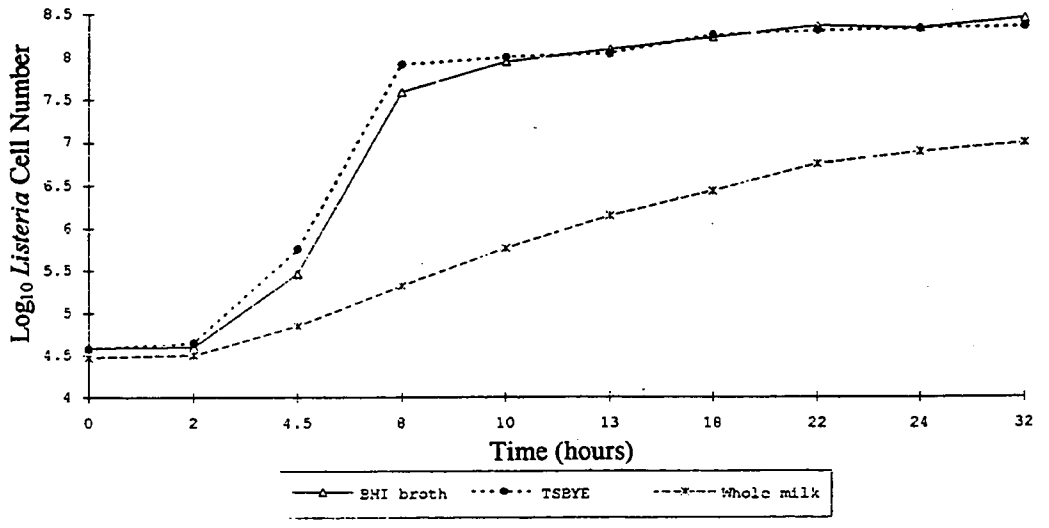


Figure 4.17 Growth cycle of *L. monocytogenes* (NCTC 9863) where the *Listeria* cells were loop inoculated into TSYEB, BHIB, and full cream milk test cultures and aerobically cultivated (110 rpm) at 37°C.

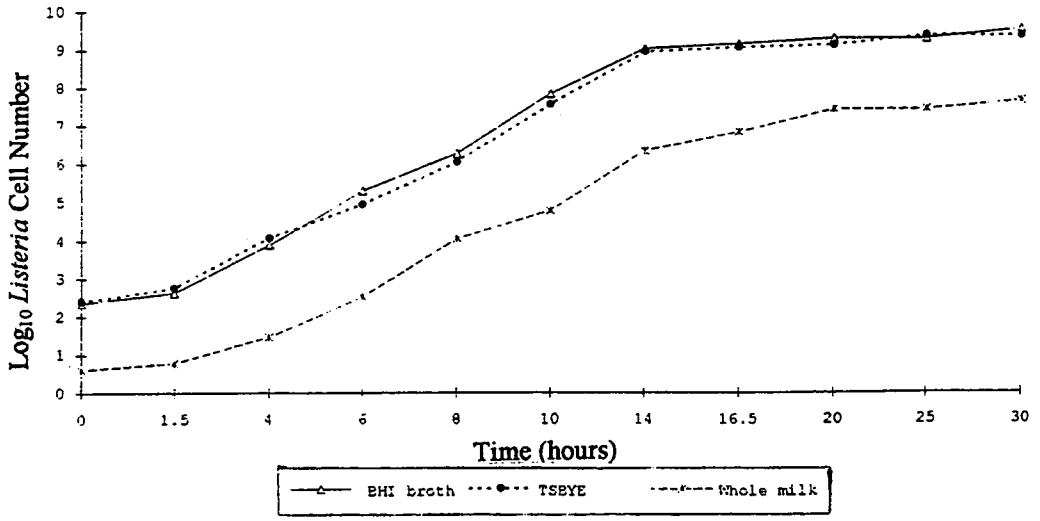


Figure 4.18 Growth cycle of *L. monocytogenes* (NCTC 9863) where the *Listeria* cells from a homogeneous broth subcultures were inoculated into complimentary TSYEB, BHIB, and full cream milk test cultures and aerobically cultivated (at 110 rpm) at 37°C.

Figure 4.17 confirmed that 20 to 24 hour old cells of *L. monocytogenes* which had been previously heat treated at 62.8°C were in the stationary phase. It also revealed that *Listeria* cells of this particular strain cultivated in autoclaved milk grew poorly compared to the other test media under study. The overall technique of subculture preparation and test culture incubation provided a fixed concentration of cells which shared similar physiological properties. The relationship between turbidity measurements (Abs /units) and total aerobic mesophilic counts (CFU ml^{-1}) for *L. monocytogenes* orbitally cultivated in TSEYB is illustrated in Figure 4.19 .

It was possible to calculate the division rate (ν) for *L. monocytogenes* cultivated in each of the test culture medium at any particular point in the cells log phase of growth by application of the formula:

$$v = \frac{\log N - \log No}{\log 2(t - to)}$$

Where No and N represents the number of *Listeria* cells present at time t 0 hours and t hours storage respectively. Indeed, this division rate can also be represented as a generation time (g) where g = 60 mins/v.

The generation time for a 10 hour test culture of *L. monocytogenes* (NCTC 9863) grown in TSYEB, BHIB and full cream milk was 36.1 mins, 35.08 mins and 37.05 mins respectively. *Listeria* exhibied lower generation times when cultivated in autoclaved milk, this reduction in growth rate may be due to the destruction of important nutrients during this sterilising process. Furthermore, *L. monocytogenes* cells cultivated in sterilised full cream milk did reach the late exponential phase to early stationary phase in its growth cycle after 20 to 24 hours orbital cultivation at 37°C and over the course of this growth cycle a significantly lower concentration of *Listeria* cells (P 0.05) was obtained in this test medium compared to the same cells cultivated in TSYE and BHI broths.

In order to render the milk totally safe for human consumption, it should under go a mild thermal process which is considered bactericidal to certain spoilage and pathogenic bacteria (except endospore formers). As the composition of milk will alter at temperatures normally associated with autoclaving (e.g. Maillard reactions), a balance between the desired and/or acceptable level of microbial kill and that of nutrient destruction must be obtained. The higher the heating temperature employed the greater will be the degree of chemical and/or nutritional alteration in addition to cell death.

The above growth cycle determination for *L. monocytogenes* (NCTC 9863) was repeated where the cells were cultivated in tyndallised (section 3.13) rather than autoclaved full cream milk (Figure 4.20). The result of this growth cycle revealed that despite the differences in initial cell concentration between the loop to broth type inoculation (~10^5 CFU ml^{-1}) and the broth subculture to broth test culture type inoculation (~10^1 to 10^2 CFU ml^{-1}), both test cultures achieved a stationary growth phase after 20 to 24 hours under the present experimental conditions.

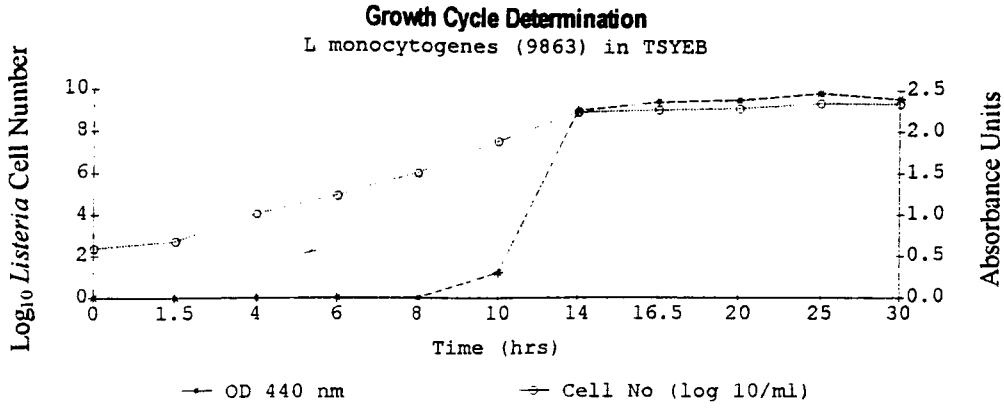


Figure 4.19 Growth cycle for *L. monocytogenes* (NCTC 9863) cultivated in TSYEB under aerobic incubation at 37°C as determined by turbidity measurements (Abs units) and total aerobic mesophilic counts (CFU ml^{-1}).

Furthermore, a 20 hour cell concentration of $>10^8$ CFU ml^{-1} and $\sim10^9$ CFU ml^{-1} was achieved for test cultures which had been inoculated by the loop-broth and broth-broth type method respectively. The results from these 2 growth cycles would suggest that tyndallisation of full cream milk is a less destructive method of heat sterilisation and creates a better growth medium compared to that of autoclaved milk. Clearly, heat labile foods such as reconstituted infant milk formulae should be tyndallised to sterility.

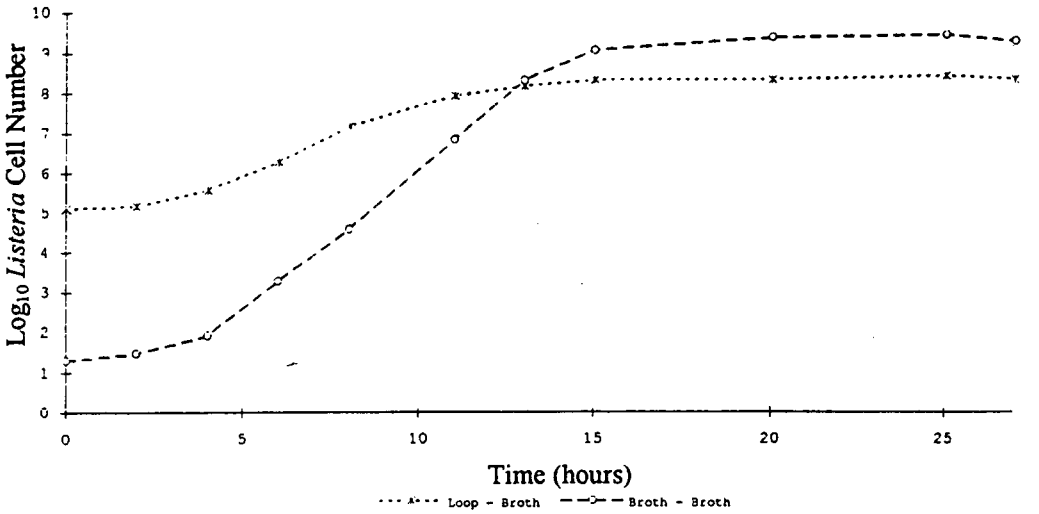


Figure 4.20 illustrates the growth cycle for *L. monocytogenes* (NCTC 9863) cultivated in loop inoculated and broth inoculated tyndallised full cream milk under orbital incubation at 37°C.

4.1.2.2.4 The effect of employing a constant medium composition throughout the cultivating, heating, diluting and enriching stages on the heat resistance of *L. monocytogenes*.

In this thermal study tyndallised whole milk (or BHI broth) was employed at each stage, (e.g. milk was used as the cultivation medium, heating menstruum, diluent and enrichment medium), in order to determine whether or not keeping the medium composition constant throughout the entire experiment would influence the heat resistance of *L. monocytogenes*. This study also attempted to establish whether whole milk provided greater recovery of healthy and thermally damaged cells compared to cells grown, heated, diluted and enriched in BHI broth. This was the first thermal investigation to employ an enrichment stage, in addition to the direct plating of heat treated *Listeria* cells.

A final concentration of approximately 10^9 *Listeria* cells ml^{-1} (NCTC 9863) was obtained in tyndalllised whole milk as described in the previous study. A 1 ml aliquot of this test culture was aseptically inoculated into a series of 28 ml McCartney bottles

containing 9 ml of tyndallised whole milk. These thermal inactivation bottles were pre-labelled as follows; H_0, H_1,H_2, H_3, H_4 (for samples removed during the heating up (H) period in mins); T_0, T_1, T_2, T_3, T_4, T_6, T_{10}, T_{20}, T_{30} (for samples removed during the holding (T) period in mins); C_1 (the 24 hour non heat treated test culture control); and C_2 (heating menstruum control).

A preliminary study was carried out to determine the rate of temperature increase in the whole milk heating menstruum and the time required for this heating menstruum (containing suspended *Listeria* cells) to equilibrate with the holding temperature of 62.8°C. The heating up period was determined by monitoring the rate of temperature increase in whole milk up to the holding period at 62.8°C via an inserted thermometer. Samples (0.1 ml) were removed at the time intervals mentioned above and immediately plated onto TSYEA and LSA plating media, plates were incubated for 72 hours at 37°C. Care was taken to ensure that all plating media were thoroughly dried (i.e. free from any visible signs of surface water) before use. Enumeration and detection of surviving *Listeria* cells was achieved via end point determination, where 0.1 ml aliquots of all the non-diluted heat treated samples were spread plated on the above plating media.

The remaining heating menstruum (plus controls) was left to enrich in a stationary position for 48 hours at 25°C prior to enumeration on TSYEA and LSA as described above. The identity and purity of the generated *Listeria* colonies was confirmed as described earlier and all plating media were visually screened for atypical colonies. The study was repeated using BHI broth instead of the tyndallised whole milk as the test medium in all the steps mentioned earlier.

Results from this study showed that enhanced recovery of healthy and heat injured cells of *L. monocytogenes* (NCTC 9863) was achieved when the organism was cultivated, heat treated, diluted and enriched in tyndallised whole milk compared to BHI broth (Table 4.6). Enrichment of cells in milk resulted in the recovery of *Listeria* colonies after 20 mins (T_{20}) holding at 62.8°C, while heat treated cells were not recovered on TSYEA plates after 2 mins (T_2) using the direct plating technique. Test cultures cells which were enriched in BHI broth after heat treatment were not recovered after 4 min (T_4) exposure at 62.8°C.

Test cultures which were heat treated in either BHI broth or whole milk and directly plated onto TSYEA plates did not tolerate more than 2 mins at 62.8°C. Indeed, direct plating without subsequent broth enrichment was a poor recovery technique for detecting sublethally injured *Listeria* cells. During the course of cultivation stage, a mechanical fault occurred with the orbital incubator where it failed to provide any rotation and the internal temperature increased to 40.2 ± 1.8°C (as detected by the maximum/ minimum thermometer). Therefore, instead of providing rotation at 110 rpm at 37°C, the cells were cultivated under static conditions (0 rpm) at the elevated growth temperature of 40.2± 1.8°C .

It would appear that either stationary cultivation and/or growth at an elevated temperature may have contributed to the enhanced recovery of *Listeria* cells. As the whole milk enriched samples provided a greater recovery of *L. monocytogenes* cells compared to cells which were enriched in BHI broth, it was probable that certain nutritional, physico-chemical constituents within the whole milk provided a certain degree of thermal protection. Furthermore, heat treated cells were statically enriched at of 25°C for 48 hours prior to isolation on TSYEA and LSA.

It has been previously shown that anaerobic enrichment is important to the recovery of heat treated *Listeria* cells, as damaged cells may be sensitive to the by-products of dissolved oxygen (e.g free radicals) formed during enrichement in certain media (Knabel et al 1990). Enrichment of healthy and sublethally injured *Listeria* cells in the long narrow McCartney bottles under static conditions (0 rpm) may have resulted in localised anaerobic conditions post heat treatment.

During this study, 5 pleomorphic surface colonies of *L. monocytogenes* (R-forms) were identified on the TSYEA (NRB1, NRB2 and NRB3) and the LSA (NRB4 and NRB5) plates. These different colony morphologies were detected on plates which were seeded with heat treated whole milk test cultures (after been held for T_6 to T_{10} mins at 62.8°C).

These R-form colonies were characterised by an undulate to irregular outer margin. Figure 4.21 illustrates the difference in morphology between the outer margin of a smooth (entire edge) and rough colony form. A Gram stain of this rough surface culture revealed that the cells were arranged singly, in pairs and in long chains.

Table 4.6 End point determination of heat treated *L. monocytogenes* (NCTC 9863) exposed to a holding temperature of 62.8°C and enumerated on TSYEA and LSA by direct plating (DP) and enrichment (EN).

Time (mins) at 62.8°C	Milk (Directly plated)[a]		Milk (Enriched)[b]		BHI broth (Directly plated)		BHI broth (Enriched)	
	TSYA	LSA	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
H0	$3.0x10^7$	$1.9x10^7$	$2.5x10^8$	$2.9x10^8$	$4.0x10^7$	$3.1x10^7$	$2.0x10^8$	$2.6x10^8$
T0	$9.1x10^1$	$1.2x10^1$	TNTC[d]	TNTC	$1.3x10^2$	$2.0x10^1$	TNTC	TNTC
T1	$1.4x10^1$	2.0x1	TNTC	TNTC	1.0x1	ND	TNTC	TNTC
T2	9.0x1	ND[c]	TNTC	TNTC	ND	ND	TNTC	TNTC
T3	ND	ND	TNTC	TNTC	ND	ND	TNTC	TNTC
T4	ND	ND	TNTC	TNTC	ND	ND	TNTC	TNTC
T5	ND	ND	TNTC	TNTC	ND	ND	ND	ND
T10	ND	ND	TNTC	TNTC	ND	ND	ND	ND
T15	ND	ND	TNTC	TNTC	ND	ND	ND	ND
T20	ND	ND	TNTC	7.0x1	ND	ND	ND	ND
T30	ND	ND	ND	ND	ND	ND	ND	ND
C1	$3.3x10^7$	$2.7x10^7$	$3.6x10^8$	$2.6x10^8$	$3.1x10^8$	$3.0x10^8$	$2.9x10^8$	$2.8x10^8$
C2	ND	ND	ND	ND	ND	ND	ND	ND

[a] samples were directly plated on TSYEA or LSA without additional enrichement
[b] samples were enriched for 3 days prior to spread plating and enumeration
[c] No colonies of *L. monocytogenes* developed at this particular sample time
[d] Too numerous to count (>300 colonies per plate)

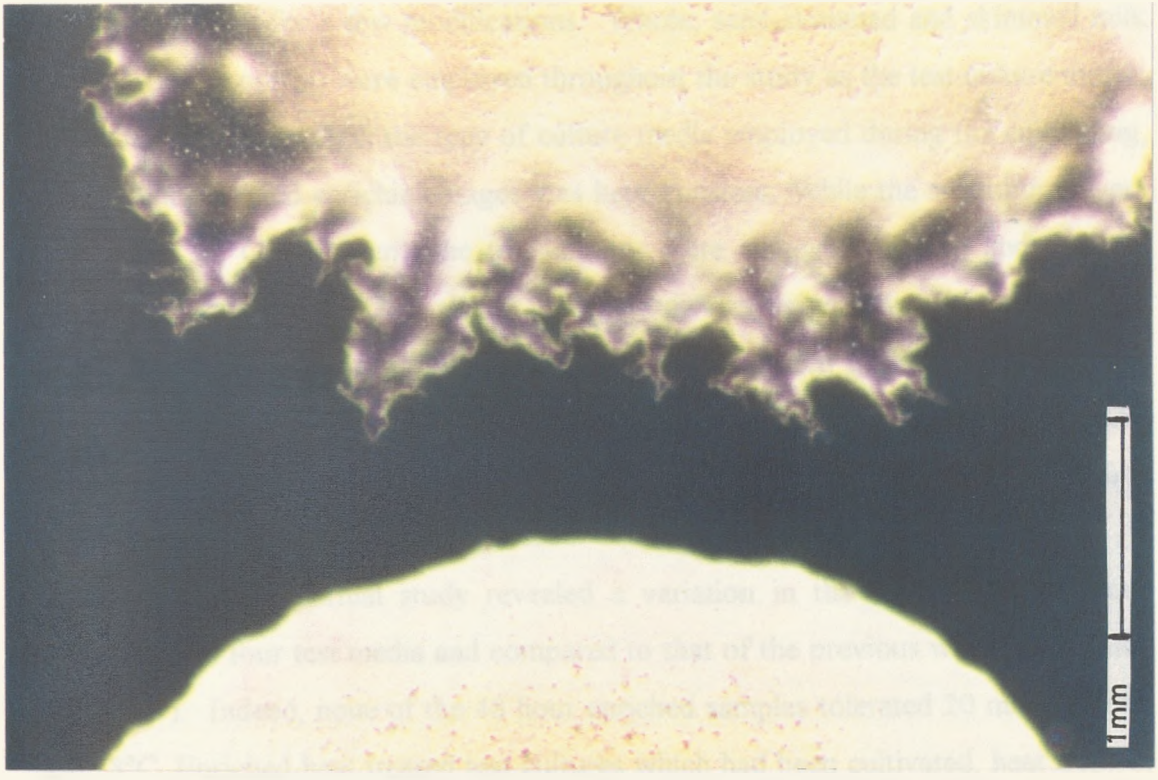


Figure 4.21 illustrates the difference in colony morphology between the outer margin of a smooth (on the left) and a rough (on the right) form of *L. monocytogenes*.

4.1.2.2.5 The effect of milk fat composition on the subsequent heat resistance of *L. monocytogenes* NCTC 9863.

Due to the recovery of *Listeria* cells at extensive holding times (~T_{20} mins at 62.8°C) where whole milk was employed as the growth medium, heating menstruum, diluent and enrichment medium, the study was repeated using whole milk, semi-skimmed and skimmed milk (and BHI broth as a control) as the test media in order to confirm the previous findings and to establish whether of not milk fat composition plays a significant role in protecting *Listeria* cells against the bactericidal action of pasteurisation. In addition, since the test culture was statically cultivated at 40.2 ± 1.8°C for part if not all of the cultivation period, the various media were statically (0 rpm) and orbitally (110 rpm) cultivated at the normal growth temperature of 37°C prior to thermal inactivation in order to establish whether agitation of the test culture influences thermal tolerance at 62.8°C

The same experimental procedure to that of the previous study (section 4.1.2.2.4) was employed, except for a few modifications. Whole, semi-skimmed and skimmed milk (and BHI as a control) were employed throughout the study as the test culture media. As for the previous study, the type of culture media employed during the cultivating, heating, diluting and enriching stages was kept constant. While the subcultures were cultivatedat 37°C (110 rpm), the test cultures were statically (0 rpm) and orbitally (110 rpm) cultured at 37°C for the 24 hour period. Thermal inactivation, direct plating and/or subsequent enrichment, and enumeration were carried out in a similar manner to that of the previous heating study. The rate of temperature increase and the time required for both semi-skimmed and skimmed milk to equilibrate with the holding temperature of 62.8 ± 0.2°C was assessed.

Results from this thermal study revealed a variation in the level of *Listeria* cell recovery in all four test media and compared to that of the previous whole milk study (Table 4.7). Indeed, none of the 48 hour enriched samples tolerated 20 mins holding at 62.8°C. Enriched heat treated test cultures which had been cultivated, heat treated, diluted in full cream milk provided the greatest level of *Listeria* cell recovery which was shown to be T_{10} mins at 62.8°C via end-point determination. The cultivation of *L.*

monocytogenes in semi-skimmed and skimmed milk at 37°C did not provide a level of heat protection greater than T_2 mins and T_5 mins holding as determined by direct plating and enrichment respectively. While the milk fat composition and/or static cultivation (and enrichment) appeared to provide greater protection against the lethal effects of pasteurisation, they alone were not responsible for the enhanced heat resistance shown in the previous heat study (section 4.1.2.2.4). This would suggest that incubation of cells at an above optimum growth temperature may influence the thermotolerance of *L. monocytogenes*. Immediate plating of the heat treated test cultures (without further enrichment) resulted in the recovery of *L. monocytogenes* cells up to and including 2 min holding at 62.8°C.

Table 4.7 The effect of milk fat composition and aerobic cultivation on the subsequent heat resistance of *L. monocytogenes* (NCTC 9863) which had been held at 62.8° C over a 30 mins period and enumerated on TSYEA.

TIME (mins)	MILK (WHOLE) *			MILK (SEMI-SKIM)*			MILK (SKIM)*			BHI BROTH*		
	DP†	EN ‡ 110 rpm	EN¶ 0 rpm	DP	EN 110 rpm	EN 0 rpm	DP	EN 110 rpm	EN 0 rpm	DP	EN 110 rpm	EN 0 rpm
H0	1.2×10^4	2.2×10^9	2.1×10^8	1.0×10^4	1.7×10^9	2.0×10^8	1.2×10^4	2.5×10^9	3.3×10^4	2.6×10^8	2.5×10^9	2.1×10^4
T0	4.0×10^1	TNTC	TNTC	8.0×10^6	TNTC	TNTC	4.0×10^6	TNTC	TNTC	6.0×10^6	TNTC	TNTC
T1	2.0×10^6	TNTC	TNTC	ND	TNTC	TNTC	ND	TNTC	TNTC	ND	TNTC	TNTC
T2	1.0×10^6	TNTC	TNTC	ND	TNTC	TNTC	ND	TNTC	TNTC	ND	3.0×10^9	TNTC
T3	ND	TNTC	TNTC	ND	ND	TNTC	ND	ND	TNTC	ND	ND	TNTC
T4	ND	TNTC	TNTC	ND	ND	TNTC	ND	ND	TNTC	ND	ND	TNTC
T5	ND	TNTC	TNTC	ND	ND	TNTC	ND	ND	ND	ND	ND	ND
T8	ND	ND	TNTC	ND	ND	ND	ND	ND	ND	ND	ND	ND
T10	ND	ND	TNTC	ND	ND	ND	ND	ND	ND	ND	ND	ND
T15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T25	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1	1.01×10^9	8.9×10^8	3.1×10^8	2.2×10^9	1.5×10^9	1.9×10^8	1.14×10^9	1.25×10^9	3.0×10^8	1.21×10^9	1.08×10^9	2.5×10^8
C2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

† Represents non-heat and heat treated test cultures which were statically cultivated prior to heating and directly plated onto TSYEA without further enrichment.
‡ Represents non-heated and heated test cultures which were orbitally cultivated, heated and then enriched prior to enumeration.
¶ Represents non-heated and heated test cultures whih were statically cultivated, heated and the n enriched prior to enumeration
* The viability of each heated test culture was determinted by end point determimation (i.e. enumerated either the directly plated or enriched sample without carrying out any additional dilutions).

Test cultures which were statically cultivated compared to aerobically incubated were statistically shown to be more heat tolerant (P 0.05). In addition, greater recovery of heat treated cells occurred following enrichment compared to direct plating (P 0.05). Under these particular sets of experimental conditions, it was not possible to determine the heat tolerance of this organism by way of a decimal reduction time ($D_{62.8°C}$ value), as a thermal death curve could not be constructed due to the holding temperature of 62.8°C being too severe (i.e. insufficient data points (log_{10} $CFUml^{-1}$) were generated at this temperature). This series of thermal studies illustrated that static cultivation and subsequent static enrichment was better than orbital cultivation for the recovery of thermally damaged cells. No pleomorphic surface colonies were identified on any of the plating media from either the non heat and/or the heat treated test cultures.

4.1.2.2.6 The effect of above optimum growth temperature and static cultivation conditions on the heat resistance of *L. monocytogenes* (NCTC 9863) exposed to a vat pasteurisation temperature regime.

The previous study was repeated using this elevated cultivation temperature (40 ± 0.3°C) in conjunction with static conditions in order to establish whether these parameters (in addition to milk fat composition) would result in an increased recovery of healthy and heat injured *Listeria* cells. The same test media were employed and each type of test media was used throughout the entire experiment procedure (see section 4.1.2.2.4).

The test culture medium was cultivated at the elevated temperature of 40± 0.3°C for 24 hours under static conditions (0 rpm). The *Listeria* cells were heat treated at 62.8± 0.2°C, enriched (statically) and enumerated (end-point determination only/ no dilutions) as described in the previous thermal study. Visual screening for variant colonial morphological forms of *L. monocytogenes* was performed on all non heat and heat treated test culture plates (visual screening occurred for all four forms of growth media).

The results of this thermal study are shown in Table 4.8, where it was found that the present combination of cultivation conditions (40 ± 0.3°C at 0 rpm), heat treatment conditions, enrichment conditions (72 hours at 30°C) and enumeration conditions (72 hours at 37°C) substantially increased the recovery of heat treated *Listeria* cells (that had been either directly plated or enriched prior to enumeration) compared to that of previous heat studies.

Table 4.8 Recovery of heat treated cells of *L. monocytogenes* (NCTC 9863) which had been statically cultivated at 40 ± 0.3°C, heat treated at 62.8 ± 0.2°C, diluted and statically enriched in the same test media, followed by enumeration on TSYEA by either direct plating (DP) with/without subsequent enrichment (EN).

TIME (mins)	FULL CREAM MILK		SEMI-SKIMMED MILK		SKIMMED MILK		BHI BROTH	
	DP†	EN‡	DP	EN	DP	EN	DP	EN
II_0	8.1×10^6	3.8×10^4	7.1×10^6	4.8×10^6	8.3×10^6	3.1×10^6	1.1×10^7	2.1×10^6
T_0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
T_1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
T_2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
T_3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
T_4	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
T_5	3.0×10^2	TNTC	TNTC	TNTC	TNTC	TNTC	1.4×10^1	TNTC
T_8	2.0×10^5	TNTC	5.0×10^5	TNTC	1.0×10^6	TNTC	ND	TNTC
T_{10}	ND	TNTC	ND	TNTC	ND	TNTC	ND	TNTC
T_{20}	ND	TNTC	ND	TNTC	ND	TNTC	ND	TNTC
T_{25}	ND	ND	ND	8.0×10^6	ND	1.5×10^1	ND	ND
T_{30}	ND	ND	ND	ND	ND	ND	ND	ND
C_1	7.6×10^7	1.1×10^4	9.1×10^7	1.6×10^4	8.7×10^7	3.6×10^4	1.9×10^8	2.2×10^4
C_2	ND	ND	ND	ND	ND	ND	ND	ND

† Represents the recovery of non-heat and heat damaged cells of *L. monocytogenes* which were directly plated onto TSYEA without either dilution or subsequent enrichment.
‡ Represents the recovery of non-heat and heat damaged cells of *L. monocytogenes* which were statically enriched for 48 hours at 30°C prior to enumeration via the above end point determination method.

Under the present set of experimental conditions, heat treated *Listeria* cells were recovered in enriched full cream milk, semi-skimmed milk, skimmed milk and BHI broth at holding times (i.e. 62.8°C) of T20 mins, T25 mins, T25 mins and T20 mins respectively. *Listeria* cells failed to survive the full heating pasteurisation cycle of 30 mins at 62.8°C, however as there were no samples taken between the holding times of

T25 mins and T30 mins, some stressed cells may have tolerated a holding time of T29 mins.

The implementation of elevated growth temperatures and static cultivation conditions have possibly contributed to the enhanced recovery of *Listeria* cells which had been directly plated onto TSYEA (i.e without additional enrichment). Indeed, heat subjected cells were recovered from full cream milk, semi-skimmed and skimmed milk and BHI broth test cultures at holding times of T8mins, T8 mins, T8 mins and T5 mins respectively via this direct plating technique. This was an improvement of approximately 6 mins (at 62.8°C) compared to the holding times tolerated by *L. monocytogenes* NCTC 9863 in previous thermal inactivation studies.

It is probable that this enhanced thermal resistance was not solely attributed to the influence of elevated growth temperature alone, rather the overall thermal resistance appears to be governed by a multitude of inter-related cultural parameters, the contribution of each being either large or small. This would explain why the determination of these critical cultural properties required a multifaceted approach. Knabel *et al.* (1990) suggested that the enhanced heat resistance shown by *Listeria* cells cultivated at temperatures at or slightly above the maximum for physiological growth may be due to the accumulation of heat shock proteins prior to heat treatment.

While directly plated cells (consisting of uninjured and sublethally damaged cells) were recovered at holding times of 8 mins, it was not possible to plot the thermal death rate for the suspended cells during this T0 mins to T8 mins holding period, as samples removed during this period were simply analysed for absence or presence of *Listeria* cells (i.e end-point determination technique) and no actual quantification of cells occurred (i.e. each sample was analysed in a non diluted state). Therefore, the only measure of thermal resistance for cells cultivated and heated in each of the 4 test cultures was to determine the end point at which damaged cells were recovered at the holding temperature of 62.8°C.

While this method of heat resistance determination was satisfactory for establishing large differences between recovery techniques (e.g. direct plating and enrichment) and it did provide a general guide as to the recovery potential for each combination of test

media, cultivation conditions etc, it did not however provide suitable thermal death rate data which would be used to establish the heat resistance capabilities of *Listeria*. Milk fat composition did not appear to enhance the recovery of heat damaged *listeria* cells by either the direct plating or the enrichment technique compared to that of the previous thermal study. In fact whole milk (cell detection at $T_{20\ mins}$) proved to be an inferior enrichment medium to that of semi-skimmed ($T_{25\ mins}$) and skimmed milk ($T_{25\ mins}$) in this particular thermal inactivation study. However, as there were no samples taken between T_{20} and $T_{25\ mins}$, it is probable that the difference in recovery potential between these test media may not be significant.

As the heat resistance of *L. monocytogenes* was determined by end-point analysis of non-diluted heat treated test cultures, very few plates supported the growth of isolated colonies (the rest were either too numerous to count or colonies simply had not grown). Indeed, as a Total Aerobic Mesophilic Count (CFU ml^{-1}) was not carried out on the heat treated test cultures at each sample time interval, isolated *Listeria* colonies only occurred on plates where the number of cells were reduced below 300 CFU ml^{-1}.

While this reduced the chances of generating rough surface cultures, a limited number of pleomorphic colonies were identified from all four test media post heat treatment (R-forms occurred on sample plates which had been directly plated and enriched). Non heat treated test cultures of *L. monocytogenes* failed to support R- form colony development. Under the present set of experimental conditions there appears to be a link between elevated growth and thermal inactivation temperatures and the emergence of pleomorphic surface cultures.

4.1.2.2.7 Confirmation that elevated growth temperature and/or static cultivation influences the thermotolerance of *L. monocytogenes* NCTC 9863.

This thermal study examined the effect of cultivation temperature and rotatory conditions prior to heating on the heat resistance on *L. monocytogenes* NCTC 9863

cells (as measured by end-point determination at the holding temperature of 62.8°C and enrichment). While the previous experimental protocol was repeated (section 4.2.2.6), certain modifications were also made. Namely, attention was focused on the test cultivation stage, where *L. monocytogenes* was only cultivated in either tyndallised whole milk or BHI broth at 37°C, 41°C and 42°C for 24 hours under orbital (110 rpm) and/or static (0 rpm) conditions prior to heat treatment at 62.8°C (use of a maximum/minimum thermometer showed that the cultivation temperatures deviated by ± 0.3°C) . The thermal inactivation bottles (containing 9ml of either milk or BHI broth and 1ml of test culture) were labelled as T_0, T_1, T_2, T_3, T_4, T_5, T_8, T_{10}, T_{15}, T_{20}, T_{30}, C_1 and C_2 and samples were not analysed from the heating up period (which was shown to be 6 mins 32 secs and 6 mins 48 secs for tyndallised milk and BHI broth respectively). The non-diluted heat treated test cultures were directly plated onto TSYEA and LSA plates, which were incubated for 72 hours prior to enumeration. The remainder of the heat treated test cultures were enriched in a stationary position for 16 and 72 hours at 30°C prior to plating.

The results from this study revealed that *L. monocytogenes* NCTC 9863 cultivated at the elevated growth temperatures of 41°C and 42°C prior to thermal treatment survived longer exposures at 62.8°C (Table 4.9). Cells which had been initially grown under the combined conditions of static cultivation (0 rpm) at 41°C and/or 42°C tolerated longer exposures at 62.8°C compared to cells which had been either orbitally or statically cultivated at 37°C or orbitally cultivated at these higher growth temperatures.

Cells which had been cultivated at 41°C or 42°C, prior to heating and enriching in whole milk were more thermo-tolerant (P0.05) compared to those grown in BHI at both 37°C and 41°C/42°C. While 16 hours enrichment at 30°C supported equal or greater recovery of heat treated cells compared to simply direct plating, stationary enrichment at 30°C for 3 day provided better recovery conditions for this heat stressed micro-organism. *Listeria* cells which had been cultivated at or above 41°C under non-rotatary conditions (prior to heat treatment), survived longer exposures at 62.8°C compared to the same cells cultivated at 37°C (0 and/or 110 rpm) or ≥41°C (110 rpm) as detected by the direct plating technique.

For cells grown in BHI broth, the greatest recovery of healthy and thermally damaged *Listeria* cells occurred under stationary cultivation conditions at the higher growth temperatures (41°C and/or 42°C), where these cells survived 15 mins exposure at 62.8°C (via 3 days enrichment at 30°C). Although a T25 min sample point was not incorporated into the heating protocol, heat treated cultures grown (41°C at 0rpm), heated and enriched (3 days at 30°C) in whole milk at best were recovered at T20 mins holding. None of the above experimental combinations permitted the survival of *L. monocytogenes* beyond 30 mins, thus pasteurisation effectively reduces a 10^8 cell concentration (CFU ml^{-1}) to a non detectable level under the present set of experimental conditions.

Rough-form surface colonies were recovered on TSYEA and LSA plating samples from test cultures which had been grown at 41°C and 42°C and subsequently heated at 62.8°C for various holding times. Again the R colony forms were characterised by an irregular to spreading outer margin, cells analysed from this outer margin were composed of single, paired and chain arrangements. Due to the limited number of plates which contained countable colonies (i.e. heat resistance was defined in terms of end-point determination at a holding temperature of 62.8°C where heat treated samples were not diluted prior to plating) it was only possible to *identify* the combination of experimental parameters which supported R-form colony development and it was not possible to statistically discredit the other experimental combinations for failing to support any pleomorphic surface colony development.

The above evidence suggests that the provision of certain key parameters: such as; elevated growth temperatures; stationary growth and enrichment conditions; extended storage of enrichment medium; extended storage of enumeration medium and employing whole milk at each stage in the thermal study can potentially increase the heat resistance capability of *L. monocytogenes* NCTC 9863. These findings, in addition to the discovery and implementation of additional parameters may further increase the cells thermotolerance to the point where low numbers of heat treated cells present in contaminated infant milk formulae may be detected.

Table 4.9 Effect of elevated growth temperature and rotatory conditions prior to heat treatment, on recovery (and heat resistance) of heat treated *L. monocytogenes* cells as detected by 16 and 72 hour enrichment (EN) at 30°C and/or direct plating (DP).

Test cultivation conditions	Test medium	Number of cells at H_0 mins† (CFU ml^{-1})	Holding time (mins at 62.8°C) at which *Listeria* cells were recovered (end-point determination on TSAYE)		
			DP¶	EN (16 hr)*	EN (3d)‡
41°C at 125 rpm	Whole milk	8.0×10^7	2	5	10
41°C at 125 rpm	BHI broth	8.6×10^7	1	5	10
41°C at 0 rpm	Whole milk	3.0×10^7	8	20	20
41°C at 0 rpm	BHI broth	2.7×10^7	5	8	15
37°C at 125 rpm	Whole milk	1.4×10^8	0	5	5
37°C at 125 rpm	BHI broth	2.4×10^8	0	4	5
37°C at 0 rpm	Whole milk	3.6×10^7	3	5	5
37°C at 0 rpm	BHI broth	4.6×10^7	2	5	5
42°C at 0 rpm	Whole milk	2.1×10^7	10	20	20
42°C at 125 rpm	Whole milk	5.9×10^7	2	5	10
Heating menstruum	BHI broth control	ND (10^{-1})	ND (0)	ND (0)	ND (0)
Heating menstruum	Whole milk control	ND (10^{-1})	ND (0)	ND (0)	ND (0)

†Represents the concentration of L. monocytogenes cells present in the heating menstruum prior to any heat treatment (H0 mins)
¶ Represents heat treated test cultures which were directly plated onto TSYEA in a non-diluted form (no enrichment stage)
* Represents heat treated test cultures which were enriched for 16 hours at 30°C prior to enumeration via end point determination.
‡ Represents heat treated test cultures which were enriched for 72 hours at 30°C prior to enumeration

4.1.2.2.8 Determination of the heat resistance of three different strains of *L. monocytogenes.*

A set of controlled experimental parameters have currently been defined which cumulatively favour the protection of *L. monocytogenes* NCTC 9863 against normally lethal temperatures. This thermal study recreated these conditions in order to determine whether or not different strains of *L. monocytogenes* shared the same level of thermal resistance. In addition, conditions shown to enhance the emergence of

pleomorphic surface cultures of *L. monocytogenes* (as a result of thermal processing) were evaluated.

Thus, having established a set of defined cultivating, heating, enriching and enumerating conditions where cells of *L. monocytogenes* NCTC 9863 have shown to be recovered after 20 mins at a heating temperature of 62.8°C, the aim of this investigation was to determine whether or not different strains of *L. monocytogenes* (e.g. NCTC 11994 and 10357) would also exhibit an increased heat resistance under the present set of heating conditions. This was the first thermal inactivation study to assess the heat resistance of *L. monocytogenes* (NCTC 11994, NCTC 10357 and NCTC 9863) in infant milk formula (i.e. SMA Gold Cap).

The test cultures were cultivated at 41°C for 24 hours in a stationary position (0 rpm) to a final cell concentration of approximately 10^8 CFU ml^{-1} and a 1 ml aliquot of each test culture was separately inoculated into a series of pre-labelled 28 ml McCartney bottles (T_0, T_1, T_2, T_3, T_4, T_5, T_8, T_{10}, T_{15}, T_{20}, T_{30},) which contained 9 ml of corresponding cultivation medium (C_1 and C_2 being non-heat treated *Listeria* and media controls respectively). A heating up period (to 62.8°C) in each type of test medium was compensated for by following the rate of temperature increase on a thermometer which had been inserted into each of the control heating menstruua.

The heating menstruum containing the suspended *Listeria* cells was heat treated for the above mentioned time intervals at 62.8°C and these heat treated test cultures were enriched for 3 days at 30°C, in addition to direct plating onto TSYEA and LSA plating media. All plated samples were stored at 37°C for 3 days prior to enumeration. The heat resistance of each strain was determined by establishing the terminal point at which *Listeria* cells were recovered, rather than a quantification of the surviving cell number (CFU ml^{-1}), at 62.8C (also known as end-point determination).

The results of this experiment revealed a variation in thermal resistance between strains of *L. monocytogenes*, with heat stressed *Listeria* cells (NCTC 11994 and 9863) sharing similar heat resistance characteristics being recovered from enriched IMF samples after 20 mins exposure at 62.8, while *L. monocytogenes* (NCTC 10357) exhibited an inferior thermotolerance to the former organisms, only tolerating 5 mins at 62.8°C when enriched in the corresponding medium (Table 4.10). This difference in

heat resistance capability between strains of *L. monocytogenes* was exhibited in most of the directly plated and all of the enriched samples. While IMF appeared to provide superior levels of heat protection compared to the same cells which were grown, heated and enriched in TSYEB, heat stressed cells of *L. monocytogenes* NCTC 9863 tolerated longer exposures at 62.8°C when they were cultured, heated and enriched in tyndallised skim milk (i.e. surviving 25 mins at 62.8°C).

Table 4.10 Variation in recovery times for directly plated and enriched cells of *L. monocytogenes* (NCTC 11994, 10357, 9863) which were cultivated, heat treated (at 62.8°C) and enriched in SMA Gold Cap, Skim milk and TSYEB.

Time (mins) at 62.8°C	NCTC 11994				NCTC 9863				NCTC 10357				NCTC 9863	
	IMF*		TSYEB		IMF		TSYEB		IMF		TSYEB		SKIM-MILK	
	DP†	EN‡	DP	EN	DP	EN	DP	EN	DP	EN	DP	EN	DP	EN
T_0	9.0×10^6	4.0×10^5	1.0×10^7	3.6×10^5	6.1×10^6	2.8×10^5	5.5×10^6	3.2×10^5	1.6×10^6	3.1×10^6	2.1×10^6	2.1×10^6	6.0×10^6	1.9×10^5
T_0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	ND	TNTC	TNTC	TNTC
T_1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	9.3×10^2	TNTC	ND	TNTC	TNTC	TNTC
T_2	3.1×10^2	TNTC	TNTC	TNTC	1.0×10^3	TNTC	TNTC	TNTC	4.0×10^4	TNTC	ND	1.4×10^2	TNTC	TNTC
T_3	8.0×10^4	TNTC	TNTC	TNTC	2.2×10^2	TNTC	3.1×10^2	TNTC	ND	TNTC	ND	ND	1.5×10^2	TNTC
T_4	ND	TNTC	2.0×10^4	TNTC	4.0×10^4	TNTC	2.0×10^4	TNTC	ND	3.0×10^4	ND	ND	5.6×10^4	TNTC
T_5	ND	TNTC	ND	TNTC	2.0×10^4	TNTC	ND	TNTC	ND	ND	ND	ND	2.5×10^4	TNTC
T_8	ND	TNTC	ND	TNTC	ND	TNTC	ND	TNTC	ND	ND	ND	ND	ND	TNTC
T_{10}	ND	TNTC	ND	ND	ND	TNTC	ND	TNTC	ND	ND	ND	ND	ND	TNTC
T_{15}	ND	TNTC	ND	ND	ND	TNTC	ND	ND	ND	ND	ND	ND	ND	TNTC
T_{20}	ND	TNTC	ND	ND	ND	TNTC	ND	ND	ND	ND	ND	ND	ND	TNTC
T_{25}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	TNTC
T_{30}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C_1	1.6×10^6	5.8×10^5	1.51×10^7	4.1×10^5	1.1×10^6	3.1×10^5	1.4×10^6	3.6×10^5	9.0×10^7	2.7×10^6	8.6×10^7	1.9×10^6	1.5×10^5	3.3×10^5
C_2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

* Represents heat treated test cultures which were directly plated onto YSYEA (without enrichment stage)
‡ Represents heat treated test cultures which were enriched for 3 days at 30°C prior to enumertion.
* Infant milk formula was SMA Gold Cap.

Direct plating of test cultures did not provide recovery of heat treated cells at a holding time greater than 5 mins, while subsequent enrichment of these samples resulted in the recovery of cells at longer holding times, such as T20 mins, T20 mins and T2 mins (in SMA Gold Cap) and T8 mins, T10 mins and T2 mins (in TSYEB) for

L. monocytogenes NCTC's 11994, 9863 and 10357 respectively. Having determined that a difference in heat resistance potentially exists among these particular strains of *L. monocytogenes* via end-point determination, it would be appropriate to generate thermal death rate kinetic values (such as D and Z values) to quantity the levels of thermotolerance in different strains. In order to obtain these kinetic values, a thermal death rate plot of Log_{10} number of cell survivors against holding times at a heating temperature which would reduce the initial cell population by 5 to 6 log cycles over the holding period is required.

4.1.2.2.9 Construction of thermal death rate curves for 3 strains of *L. monocytogenes*.

A variation in thermotolerance among 3 strains of *L. monocytogenes* emerged in section 4.1.2.2.8, but rather than simply detecting the presence or absence of surviving cells by the end point determination technique, the objective of this study was to construct a thermal death rate time curve (TDTC) for each of these strains so that the thermal resistance capability of each strain could be quantified. A TDTC is obtained by plotting the number of cells survivors (log_{10}) against holding time at a suitable heat inactivation temperature. It is possible to obtain a thermal death rate plot at a holding temperature of 62.8°C if sufficient data points (in the form of total aerobic mesophilic counts) are obtained from the holding period.

However, this would probably mean obtaining and analysing 5 to 6 samples from a 2 min holding period. This problem can be solved by selecting a less severe heating temperature (<62.8°C), where sufficient samples can be taken over a longer holding period.

The recommended temperature for the reconstitution of infant milk powder is known to be approximately 56°C (section 2.3). Moreover, Mackay *et al.* (1989) revealed that a holding temperature of 56°C will reduce a *L. monocytogenes* cell population by 1 log_{10} fold every 14 mins (approximately). Therefore, under the present set of experimental conditions, a 4 to 5 log_{10} fold decrease in initial cell number (No) should be achieved over a 60 mins holding period at 56°C. By employing a lower thermal

inactivation temperature (e.g. 56°C) it will be possible to reduce the number of *Listeria* cells killed during the heating up period and therefore provide a large concentration of cells at T0 mins (the point of equilibration at the holding temperature). In addition, since a less severe holding temperature is employed then the cells will die off at a slower rate. A plot of the $\log_{10}$ cell number of survivors against the corresponding holding times at this reduced temperature should provide a TDTC for each strain of *L. monocytogenes* and from which, the decimal reduction time ($D_{56°C\ mins}$) can be calculated.

Indeed, Mackey *et al.* (1989) estimated that the time required to reduce a fixed cell concentration of *L. monocytogenes* by 1 log fold at 56°C (a 1 D value reduction) was approximately 14 mins, this author also illustrated the D value at 63°C to be every 0.7 mins and therefore, in 3 mins at this holding temperature a 5 $\log_{10}$ cell reduction is achieved. (this would agree with the time required to reduce a cell population to a non detectable level in previous thermal studies ,section 4.1.2.2.8). Therefore if samples are taken every 15 mins over a 60 to 90 mins holding period at 56°C, and every minute over a 5 minute holding period at 62.8°C, sufficient data points should be obtained for the purpose of constructing a thermal death rate curve (log10 cell number of survivor against holding time) at 56°C and 62.8°C.

In order to assess the level of cell recovery and the heat resistance capability under that particular set of conditions, a total aerobic mesophilic count of the heat treated test cultures were carried out on either a nonselective or selective plating media immediately after the heat exposure (i.e. heat subjected cells were not be enriched prior to enumeration).

The study was initiated by orbitally cultivating (110 rpm) 3 strains of *L. monocytogenes* (NCTC 11994, 9863 and 10357) in separate BHI broths at 37°C over a 24 hour period (single colonies of all three strains were initially obtained and their identity confirmed as described previously, section 4.1.2.1.1). A 1ml aliquot of each 24 hour adjusted $_{440nm}$ subculture (using the 10^{-4} dilution) was inoculated into separate test media (i.e. SMA Gold Cap) and then cultivated at either 41°C or 37°C for 24 hours in a stationary position. No other test media were employed in this thermal resistance study. One ml of each 24 hour test culture was then inoculated into a series

of 28 ml bottles containing 9 ml of tyndallised SMA Gold Cap (i.e. comprised the heating menstruum).

Each duplicate test culture was then heated to and held at 56°C (or H_0, T_0, T_{15}, T_{30}, T_{45}, T_{60} mins) or 62.8°C (for H_0, T_0, T_3, T_5 mins), while a non heat treated test culture (C_1) and a sterile heating menstruum (C_2) control were also employed. The sample were removed at these regular intervals and placed on ice.

Each of these dilutions were spread and spirally plated onto triplicate TSYEA and LSA plates (again care was taken to ensure that all the plates were thoroughly dried before use). In addition, 1 ml of these upper dilutions were pour plated into molten TSYEA plates in order to quantify healthy and heat stressed cells at concentrations < 10 CFU ml^{-1} .

Confirmation of cell reduction at each holding period was achieved by plating out (spirally and spread plating techniques) these non-heat and heat treated test cultures in diluted samples onto TSYEA and LSA plates. This contrasted from the previous thermal studies where a non diluted sample from each time period was spread over the surface of an agar plate and the heat resistance capability was then determined by the absence or presence of *Listeria* colonies at a particular holding time period. No enrichment of heat treated test cultures took place and all plates were incubated for 3 days at 37°C prior to enumeration. Plates were also screened for pleomorphic surface colonies of *L. monocytogenes*. Thermal death rate time curves were constructed from the plot of $\log_{10}$ cell number of survivors against holding time (mins) at either 56°C or 62.8°C.

Examination of Figures 4.21 and 4.22 shows that the experimental design (i.e. combination of heating temperatures and sampling times at 56°C and 62.8°C respectively) did provide the desired level of cell reduction (CFU ml^{-1}) in order to construct a TDTC. These thermal death rate plots of $\log_{10}$ number of cell survivors versus holding times at 56°C and 62.8C resulted in the production of thermal death rate time *curves* for all strains rather than a linear $\log_{10}$ cell death rate. It would not be theoretically accurate to calculate the D and Z thermal death rate kinetic values under these circumstances as a logarithmic (linear) death is required in order to calculate these values. Therefore, a linearisation of these curved thermal death rate plots will be

required in order to obtain tangible values (analagous to the D and Z values generated from normal linear death rates) which emphasise the heat resistance capability of each *L. monocytogenes* strain.

However, an observation of thermal death rate curves suggests that a variation in heat tolerance capability between different strains of the same organism exists and that the cultivation of all *L. monocytogenes* strains at the elevated growth temperature of 41°C prior to heat treatment provided greater recovery of heat damaged cells. In addition, *L. monocytogenes* NCTC 10357 appeared to be less heat resistant compared to the other two serovar 4b strains employed. This difference in thermotolerance was illustrated by a ~ 6 fold reduction in $\log_{10}$ cell number for strain 10357 compared to only a 2 $\log_{10}$ cycle reduction for strains 11994 and 9863 cultivated at 41°C prior to holding at 56°C for 60 min (Figure 4.21).

All strains of *L. monocytogenes* cultivated at 37°C prior to heat treatment at 56°C were recovered in lower numbers on TSYEA and LSA at each heat exposure time compared to cells cultivated at 41°C. Although the decimal reduction time values could not be calculated from both figures, however, it appeared that *L. monocytogenes* strains 11994 and 9863 exhibited similar heat resistance. Furthermore, strains 11994 and 9863 cultivated at 37°C were still more heat resistant than strain 10357 which had been cultivated at 41°C prior to heating at 56°C and/or 62.8°C. *Listeria monocytogenes* strain 10357 appeared less thermotolerant, and cultivation temperature significnatly influence the level of cell recovery at the heat inactivation temperature of 62.8°C.

Listeria cells were inactivated at a faster rate when heated to and held at the higher temperature of 62.8°C (Figure 4.22). However, a similar trend in heat resistance characteristics emerged at 62.8°C to that of the lower heat inactivation temperature, i.e. variation in strain heat resistance, superior recovery of heat damaged cells when cultivated at 41°C as opposed to 37°C prior to heating). Therefore, these findings cannot be attributed to holding the cells at a particular heating temperature.

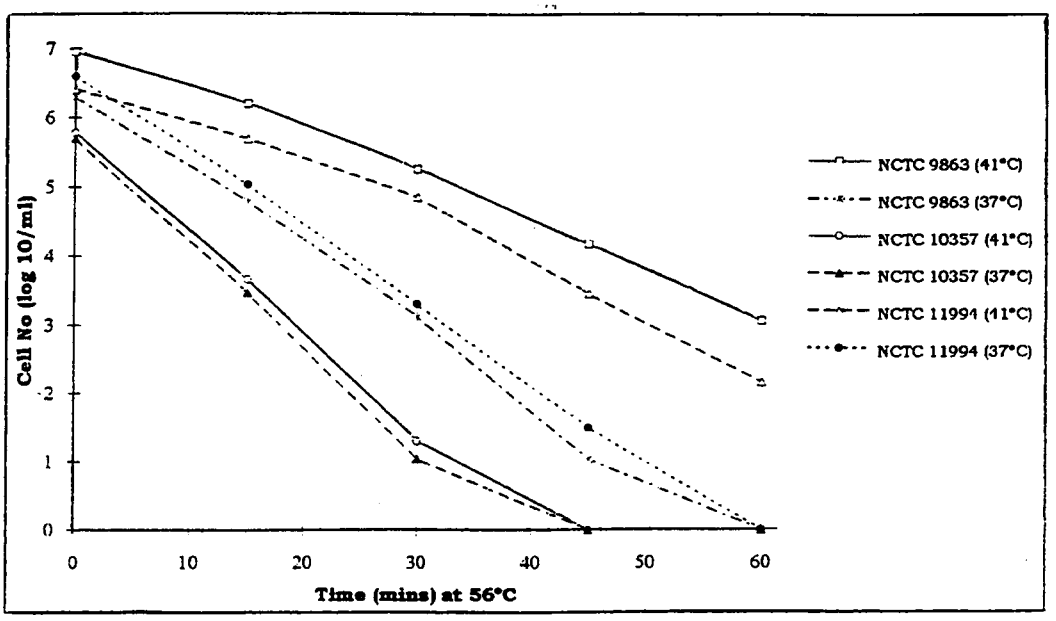


Figure 4.21 Thermal death rate *curves* [number of cell survivors (CFU ml^{-1}) on TSYEA plates versus holding time (mins) at 56°C] for 3 strains of *L. monocytogenes* which had been cultivated at 37°C and 41°C prior to heat treatment.

While the cell concentration in each flask was standardised at H_0 mins (i.e. the actual cell concentration for each strain prior to warming-up period), due to differences in heat resistance capabilities (resulting from differences in strain type and temperature of cultivation prior to heating) and the lethal effects of the warming up period variations in cell numbers were observed at T_0 mins. The variation in cell concentration obtained as a result of the bactericidal effects of the warming up (H0 to T0 mins) and holding (T0 mins to either T30 mins or T5 mins) periods appeared to follow a similar trend.

A greater recovery of heat treated *Listeria* cells were obtained on the non-selective TSYEA plating media and the longer the exposure either 56°C or 62.8°C, the greater the difference in Total Viable Counts (CFU ml^{-1}) between this plating medium and LSA (Tables 4.24 and 4.25 in appendix). The reason of this significant difference in the level of *Listeria* cells recovered may be due to TSYEA supporting the growth of healthy and sublethally injured cells, while the LSA plating medium may have recovered uninjured and a small number of heat damaged cells (if any).

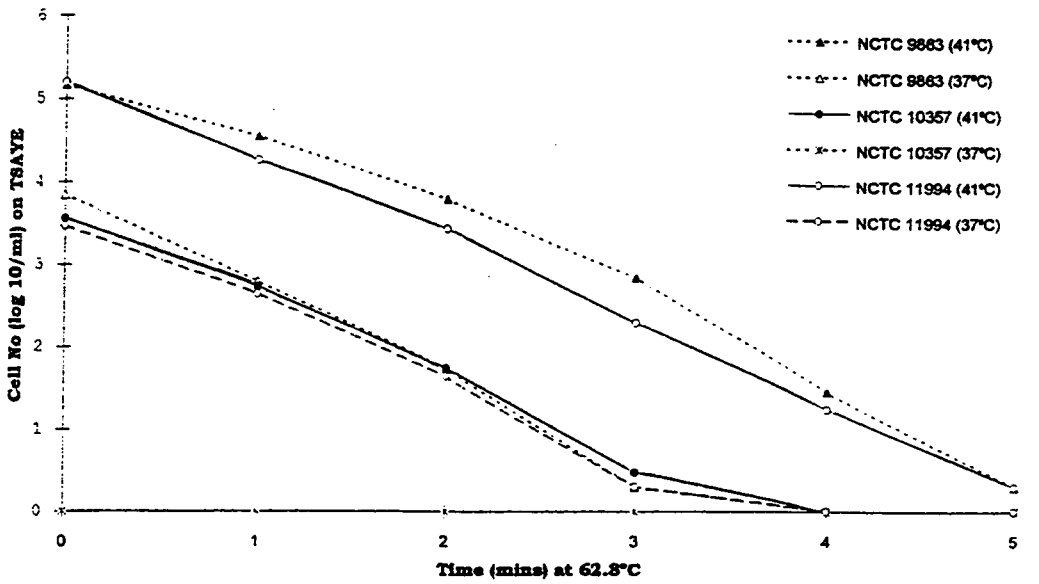


Figure 4.22 Thermal death rate *curves* [number of cell survivors (CFU ml^{-1}) on TSYEA plates versus holding time (mins) at 62.8°C] for 3 strains of *L. monocytogenes* which had been cultivated at 37°C and 41°C under stationary conditions prior to heating at 62.8°C.

In order to quantify the heat resistance capability of each strain and confirm the above findings, it is necessary to linearise the thermal death rate curves so as to calculate analagous values to that of the D and Z values. Linearisation of these log_{10} survivor curves is theoretically possible via the application of the formula $(\log No - \log N)^a = kt + c$ which is applied in the next section.

Pleomorphic surface colonies similar to those previously generated in other thermal studies were obtained on TSYEA and LSA plates from heat treated test cultures of *L. monocytogenes* (NCTC 11994, 9863), however, the less heat resistant 10357 strain failed to produce any of these R-form colonies.

4.1.2.2.10 Linearisation of the thermal death rate curves derived from heat treated test cultures of *L. monocytogenes.*

Death is defined as the loss of the cells ability to reproduce when, as far as known, suitable conditions for reproduction are provided (Schmidt 1954). Microbial cell death can be brought on by the exposure of cells to a lethal agent such as heat. Heat is a molecular energy state that is capable of producing changes in the cell which prevent the cell from reproducing by either direct effects on the reproductive mechanism and/or by disrupting cellular metabolic systems that provide energy and chemical intermediates for reproduction (Block 1955).

The thermal death rate of micro-organisms very often is assumed to be logarithmic, i.e. the result of the thermal rate plot of $\log_{10}$ cell number of survivors against holding time is a straight line. One of the most commonly employed mathematical measures of cell destruction during heating is the D value, which is the time required (at a particular temperature) to reduce the surviving fraction of organisms by 10 fold. At a constant D value, the time for a given probability of inactivation is directly proportional to the initial number of organisms present. Having determined a range of D values at different heating temperatures , a Z value for that particular organism can then be calculated. The Z value is the number of degrees (e.g. °C) required to alter the D value 10 fold or to traverse 1 log cycle. Computation of D and/or Z values can only occur when a *straight* line thermal death rate plot has emerged.

Experimental deviations from this linear or logarithmic order of thermal death occur frequently and are usually explained by citing factors which influence the heat activation of the organism; non-uniformity of microbial populations or inadequacies of the heating procedure. Calculations of lethalitys from these non-linear curves have proven to be complicated.

From the results of previous thermal inactivation studies it has been demonstrated that cells of *L. monocytogenes* exhibit a non-linear (i.e. curved) thermal death rate when held at 56°C and 62.8°C under a defined set of heating conditions. Therefore, death rate data for *L. monocytogenes* deviate from the logarithmic order of death. In particular, at the lower heating temperatures (e.g. 56°C) these log survivor curves

were characterised by a shoulder proceeding an accelerating death rate, while at higher lethal temperatures (e.g. 62.8°C) this shoulder effect may be masked by the severity of the treatment or by the inability of the experimental technique to detect it. Alderton and Snell (1970) introduced a linearization for this type of death rate curve that enabled calculation of the temperature dependence for heat survival of spores at a water activity level of 0.3. This study attempts to apply this procedure to the survival curves for *L. monocytogenes* as the cells apparently exhibit lags in the biological response to heat treatments.

Moreover, this section describes how to linearise a curved thermal death rate derived from a plot of $\log_{10}$ number of cell survivors against holding time (mins at a lethal temperature) by application of the linearisation formula $(\log No - \log N)^a = kt + c$ (King et al 1979). No and N are the initial and surviving number of cells at time t. The thermal death rate is represented by k and c is a constant for the set of data. The a value (exponent) is derived form the least squares slope of the plot of log(logNo-logN) against log time and is used to linearise thermal death rate curves. The formula is simple to operate and can be employed to calculate processes for death rate curves with shoulders just as D and Z values are used in logarithmic death rate kinetics. Moreover, the formula permits calculation of other parameters analagous to those for logarithmic death (D and Z values). In the linearisation equation, k (the death rate constant) is some what like D in function assuming $c = 0$:

The actual procedure for linearisation of survivor curves is summarised as follows:

1. Construct a thermal death rate plot of $\log_{10}$ number of cell survivors against the corresponding holding times (mins) at a known bactericidal temperature and determine whether the resulting thermal death rate fits a straight (logarithmic) or a curved line.
2. If this thermal death rate fits a straight line then determination of the cells heat resistance should be approached by derivation of the logarithmic D and Z values. However, if the thermal death rate exhibits a curve (e.g. initial shoulder effect or full curve), then this curve must be linearised in order to calculate the actual time required for a 1 log cycle reduction in cell number (1/k value).

3. Linearisation of the $\log_{10}$ survivor curve is obtained by application of the formula $(\log No - logN)^a = kt+c$ [where No and N are the initial and surviving number of *Listeria* cells at time t. The thermal death rate is given by k and c is a constant for the set of data. The exponent value *a* is derived from the least squares slope of the plot of log(logNo-logN) against log holding times and is employed to linearise the thermal death rate curves.
4. Determine the exponent value *a*, (which is the reciprocal of the slope of log(logNo-logN) against log time values) by:

- converting the cell concentration (CFU ml^{-1}) at each holding time interval to its corresponding $\log_{10}$ value.
- subtracting each logN value from the initial logNo cell concentration (i.e. the concentration of *L. monocytogenes* cells present at the initial point holding at a lethal temperature - T_0 mins).
- obtaining the log of these (logNo-logN) values.
- finally plotting the log(logNo-logN) against log time values and determine *a*, which is the reciprocal slope of the plotted line.

5. Multiply the (logNo-logN) values by this newly derived exponent *a* value.
6. Construct a plot of $(LogNo-logN)^a$ against holding times and calculate the intercept *c* and the slope *k* (which is equal to the thermal death rate constant).
7. Calculate the reciprocal k value, which is an analagous parameter to that of the decimal reduction time (D) value and as such will illustrate the time required for a reduction in cell concentration (at T0 mins) by 1 log cycle.
8. It is possible to determine other k values by interpolation of the plot, log k values (from a variety of lethal holding temperatures) against their respective holding temperatures. Indeed, the plot of log k values against holding time can be employed to calculate the amount of °C rise in temperature required to reduce the 1/k values by 1 log cycle or 10 fold (i.e. analagous to that of the normal Z value).
9. Furthermore, calculation of the holding time for a fixed reduction in *L. monocytogenes* cell number can be achieved by rearrangement of the linearisation formula:

$$^{a}t = \frac{(\log No - \log N)^{a}}{k} - \frac{c}{k}$$

Thus, by simply substituting in the reduction in cell number (logNo - logN), calculation can be made of the holding time (e.g. mins) required to achieve this cell reduction without having to refer to the plot of $(logNo - logN)^{a}$ against holding time.

$$\frac{1}{k} = \frac{t}{(\log No - \log N)^{a}} \quad \text{and} \quad D = \frac{t}{(\log No - \log N)}$$

[Non -logarithmic death rate kinetics] [logarithmic death rate kinetics]

The log k values against temperature plot can be used like a Z plot to interpolate between different heating temperatures.

The rest of this section demonstrates how to apply the linearisation formula $(logNo-logN)^{a} = kt+c$ to straighten a set of thermal death rate curves produced by *L. monocytogenes* NCTC 11994 in the previous heating study (section 4.1.2.2.9). This serotype 4b strain was cultivated in SMA Gold Cap (IMF) at 41°C in a stationary position prior to heat treatment at 56°C and 62.8°C. Samples were removed from the heat treated test cultures after the designated holding time and enumerated on TSYEA and LSA plates by the direct plating technique. For the purpose of simplicity, only the total aerobic mesophilic counts (CFU ml^{-1}) obtained from the TSYEA plates were used in this demonstration.

The linearisation procedure was initiated by determining whether or not to treat the plot of $\log_{10}$ *Listeria* cell survivors against holding time as either logarithmic (best fit straight line) or non-logarithmic (a curved line) data, by constructing a graph of these parameters. If it appeared that the plot (through all the experimental points) fitted a straight line, then the order of cell death was considered logarithmic and as such, the thermal death rate kinetic values D and Z were calculated. If on the other hand, a curved thermal death rate plot was evident, then the data was treated as non-logarithmic and the formula $(logNo-logN)^{a} = kt+ c$ was applied to linearise this cell survivor curve.

Cells of *L. monocytogenes* NCTC 11994 exhibited a thermal death rate curve on heating at 56°C and 62.8°C, therefore, the data must be linearised in order to determine the heat resistance of the organism (Figure 4.23). At the temperature of 56°C a lag or shoulder preceeded the accelerated death rate portion, whereas at the holding temperature of 62.8°C, a less dramatic shoulder was evident and the death rate plot appeared almost straight. As mentioned earlier, this curvilinear portion of the survivor curve may have been masked by the severity of the heat treatment (i.e. the higher the holding temperature the less dramatic the death curve) and by the inability of the common experimental techniques to detect it.

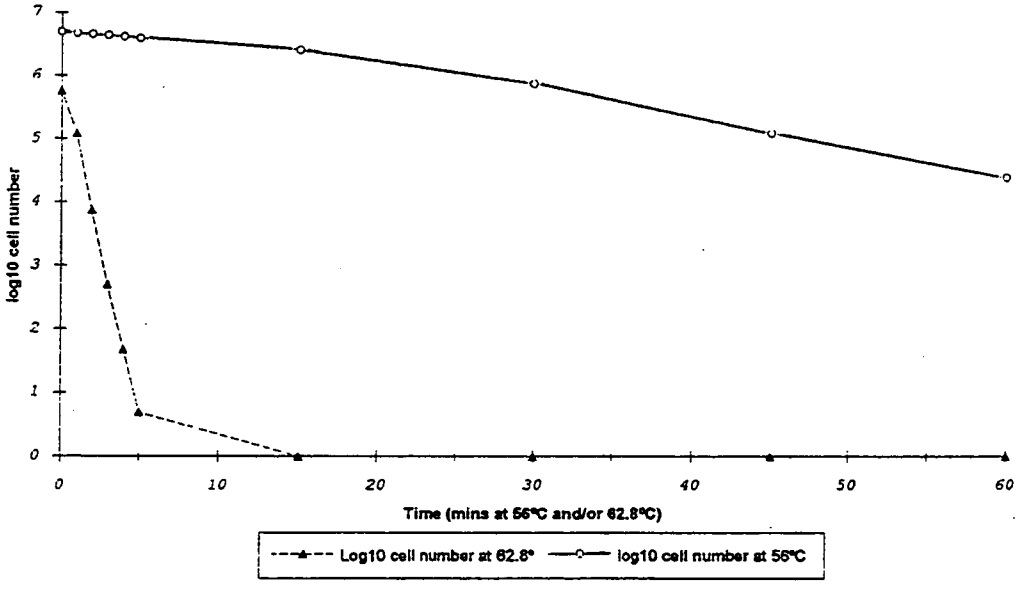


Figure 4.23. Thermal death rate curves (log_{10} cell number of survivors against holding time values) for a fixed cell concentration of *L. monocytogenes* NCTC 11994 heat treated at 56°C and 62.8°C.

The formula $(logNo-logN)^a = kt+c$ was then adopted to linearise the *Listeria* survivor curves produced as a result of heating at temperatures of 56°C and 62.8°C. The

reductions in cell concentration (CFU ml^{-1} at N) produced as a result of heating at 56°C were converted to the corresponding log_{10} values (Table 4.11). Each logN value was then subtracted from the logNo concentration of cells (i.e. the concentration of cells achieved at the point of equilibrium at 56°C =T0 mins). Where No and N are the initial and surviving number of cells at time t. The log of these logNo-logN values and the log of their corresponding holding times was then obtained. The exponent "*a*" value was determined from the plot of log(logNo-logN) against log time values, the reciprocal of the least squares slope of which gave this *a* value. In this instance the value of *a* (which would eventually be used to linearise survivor curves) was 0.62 (Figure 4.24).

Having determined the *a* value to be 0.62, linearisation of the thermal death rate curve proceeded by multiplying the (logNo-logN) by this exponent value *a*, followed by plotting these $(logNo-logN)^a$ values against the respective holding time. The intercept of the Y axis provided the c value, whereas the slope of this straight line plot gave the thermal death rate constant k value.

Table 4.11 Calculation of the exponent a value by manipulation of thermal death rate data for *L. monocytogenes* NCTC 11994 (which had been initially cultivated at 41°C under stationary conditions prior to heating at 56°C and enumeration on TSYEA plates.

Time held at 56°C (mins)	Log of time (mins)	(logNo-logN)† cell survivors	log(logNo-logN) cell survivors	$(logNo-logN)^{0.62‡}$
0	0	0	0	0
1	0	0.02	-1.699	0.088
2	0.301	0.03	-1.523	0.1137
3	0.477	0.07	-1.154	0.192
4	0.602	0.08	-1.097	0.209
5	0.698	0.10	-1.000	0.239
15	1.176	0.29	-0.533	0.467
30	1.477	0.81	-0.092	0.877
45	1.650	1.59	0.201	1.330
60	1.778	4.40	0.403	1.850

† No and N are the initial and surviving number of cells at time t.
‡ 0.62 is the exponent a value for *L. monocytogenes* NCTC 11992 for this set of experimental conditions

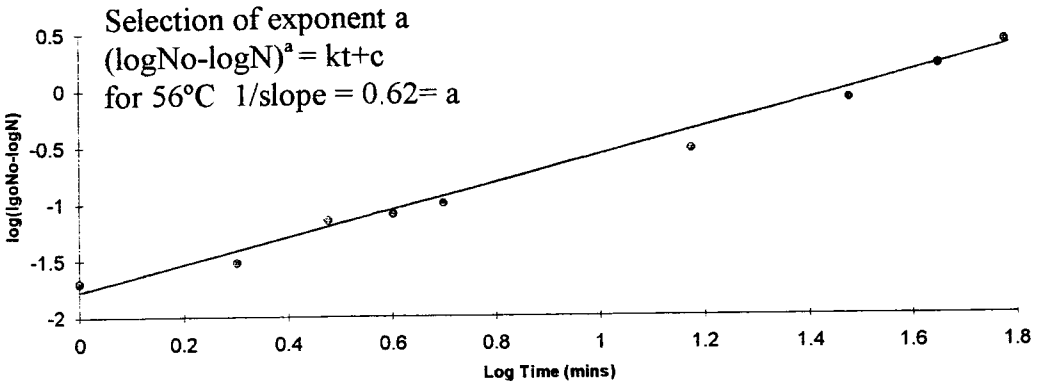


Figure 4.24 Determination of slope and exponent (value "*a*") for linearisation of death rate curves via the plot of log(logNo-logN) against log holding time (mins) values at 56°C.

Application of $(logNo-logN)^{0.62}$ values against holding times at 56°C yielded the thermal death rate constant k (0.0308) and a constant c (-0.02). If the death rate followed the expression $(logNo-logN)^a = kt+c$ exactly and there were no experimental errors at all, then c would be zero. When calculating a family of survivor curves (e.g. at 56°C, 60°C and/or 62.8°C), the *a* value from the least severe heat treatment (e.g.56°C) should be used to linearise all the death rate curves. Figure 4.25 showed that the application of the exponent *a* (0.62) in the plot of $(logNo-logN)^a$ against time did convert a thermal death rate curve to a straight line (i.e. linearisation of death rate curve for a heating temperature of 56°C occurred by application of the above expression). If the plot of log (logNo-logN) against log time had provided an *a* value equal to 1.0, then the thermal death rate would be logarithmic and D and Z values should be calculated. As the a value was illustrated to be 0.62, then cells of *L.*

monocytogenes (NCTC 11994) heated at 56°C exhibit a curved death rate (where a shoulder portion preceeds an accelerating death rate).

In the linearisation formula, k is the death rate constant for that particular microorganism which had been cultivated, heated and enumerated under a particular set of conditions. Moreover, this k value is exclusive to that particular set of experimental conditions, so if any modification in experimental design were to take place then this k value would probably change. Indeed, the reciprocal of the thermal death rate constant k value is analogous in function to that of the logarithmic D value (assuming c=0), where $1/k = t/(\log No - \log N)^a$ [$D = t/(\log No - \log N)$]. Thus, under this set of defined experimental conditions, it took 32.47 mins to reduce the *Listeria* cell population by 1 log fold (i.e. 1/0.0308 = 32.47 mins at 56°C).

The $k_{56°C}$ value, determined from the slope of the linearised curve at a heating temperature of 56°C, and the intercept or c value can be employed to calculate holding times for a given or desired amount of cell destruction by rearranging the linearisation formula $(\log No - \log N)^a = kt+c$ to the expression:

$$ {}^{a}t = \frac{(\log No - \log N)^{a}}{k} - \frac{c}{k} $$

The application of this rearranged formula will provide the *calculated* thermal death times (^at) for a desired level of cell inactivation. Indeed, the degree of cell destruction estimated as these calculated thermal death holding times can be compared to the *actual experimental* holding times used in the heat treatment of *Listeria* cells (Table 4.12).

Once the thermal death rate data had been linearised, calculation of the correct holding times (i.e. ^at) for the actual reductions in *Listeria* cell concentration was achieved by use of the above rearranged formula and the thermal kinetic values k , c and a. This calculated cell reduction time (at 56°C) can be compared to the actual experimental times used. For example, a 1.59 $\log_{10}$ cell number reduction (i.e. logNo-logN) occurred at the experimental holding time of 45 mins at 56°C, whereas the calculated holding time (on the linearised survivor curve) for the same level of cell inactivation was illustrated to be 43.93 mins.

Table 4.12 Calculation of holding time for different inactivation levels of *L. monocytogenes* NCTC 11994 compared with time estimated from experimental data (where k =0.0308, c =-0.02 and a= 0.62).

Cell reduction (logNo-logN)	t (mins at 56°C)†	
	Calculated	Experimental
0	0	0
0.293	15.16	15
0.810	29.14	30
1.590	43.93	45
2.690	60.61	60

† $t = \frac{(\log No - \log N)^a}{k} - \frac{c}{k}$

By calculating the coefficient of determination (r^2) between the calculated and the experimental holding times it was possible to illustrate how close these experimental and calculated points fitted or correlated (in this instance $r^2 = 0.993$). Under the same experimental conditions, the *a* value obtained from the least severe heat treatment should be employed to linearise all curved thermal death rate plots produced by the same organism at higher heat inactivation temperatures. It is incorrect to calculate a new *a* value for each heat treatment temperature where the only modification to the experimental protocol was the heat inactivation temperature.

Once the survivor curve for *L. monocytogenes* NCTC 11994 (which had been produced as a result of heating to and holding at 56°C), had been linearised, it was hoped that re-application of this *a* value of 0.62 would linearise the thermal death rate curves resulting from heat treating at other lethal temperatures. However, this was not the case as the plot of $(\text{logNo-logN})^{0.62}$ against holding times at 62.8C still produced a survivor curve for cells of *L. monocytogenes* NCTC 11994 (Figure 4.26). Instead, the linearisation formula $(\text{logNo-logN})^a = kt+c$ had to be re-applied to the thermal death rate data (at 62.8°C), where the plot of log(logNo-logN) against log time (secs)

values produced a new *a* value of 0.792. Subsequently, from the plot of (logNo-logN)$^{0.79}$ against holding time value, a thermal death rate constant value ($k_{62.8°C}$) of 0.0119 and an intercept of 0.15 were established.

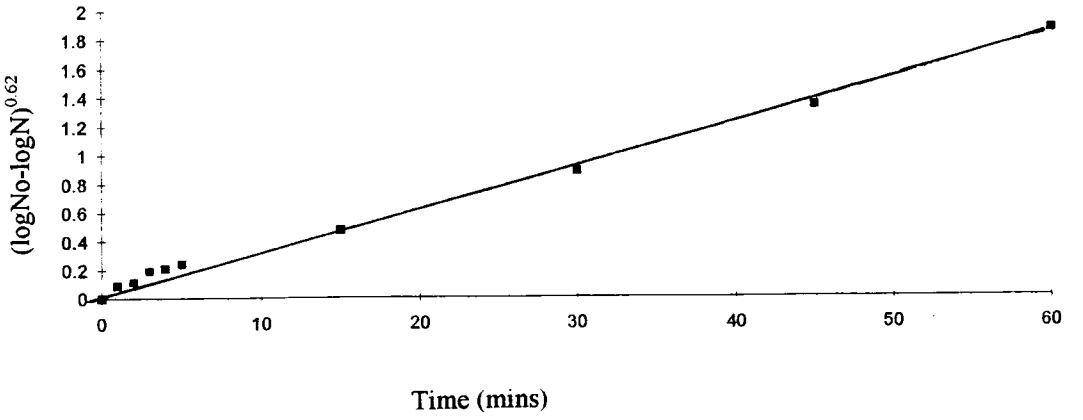


Figure 4.25 Linearisation of the thermal death rate curves exhibited by *L. monocytogenes* NCTC 11994 at a heating temperature of 56°C, by the formula (logNo-logN)^a = kt+c.

Application of the linearisation formula (having determined the k, c and a values to be 0.0119, 0.15 and 0.79 respectively) to the thermal death rate data which had been produced by cells of *L. monocytogenes* heated at 62.8°C, resulted in the straightening of the survivor curve.

The reciprocal of this thermal death rate constant (1/k) gave a value of 1.4 mins, which was equivalent to the time required to reduce the cell population by 1 log fold under the present set of experimental conditions.

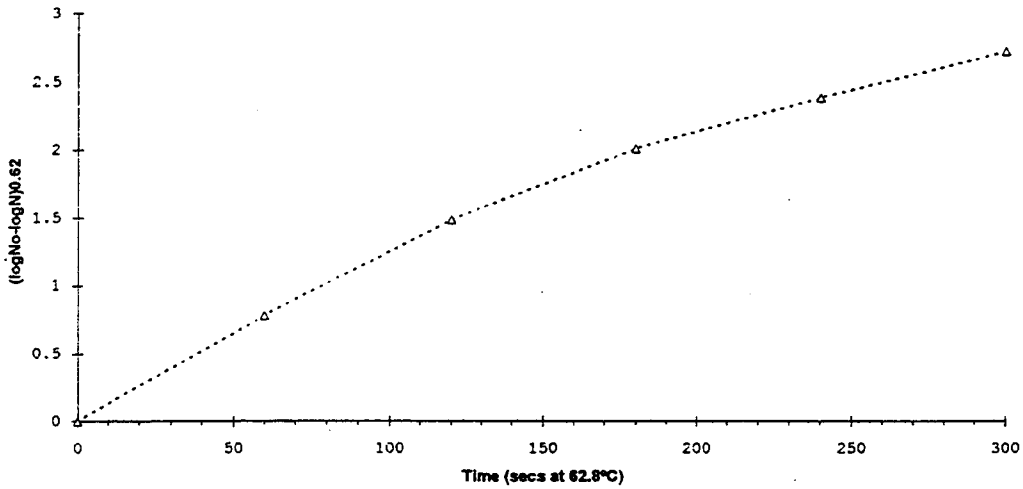


Figure 4.26 Non-linearisation of the survivor curve for *L. monocytogenes* NCTC 11994 by way of $(\log No - \log N)^{0.62}$ against holding times at 62.8°C.

The calculated thermal death rate holding times (^at) were determined from the linearised survivor curve at the heating temperature of 62.8°C by manipulating the linearisation formula as described earlier. The calculated thermal death rate times were then compared with the actual experimental holding times (interpreted from a curved plot of $\log_{10}$ cell number of survivors against time) for the same level of cell destruction (Figure 4.27). A coefficient of determination (r^2) of 0.991 was computed for the calculated and experimental holding times which had provided the same degree of cell destruction at 62.8°C.

Re-arrangement of the linearisation formula $^at = \frac{(\log No - \log N)^a}{k} - \frac{c}{k}$ permitted the calculation of the holding time for a fixed reduction in cell number by taking into consideration the shoulder portion of the survivor curve. For example, an experimental (actual) holding time (t) of 180 seconds at 62.8°C illustrated a 3.06 log cell number reduction, whereas the calculated time (^at) for the same degree of cell inactivation was 215.92 seconds. The calculated thermal holding times evenly distribute the thermal death rate over the full holding period.

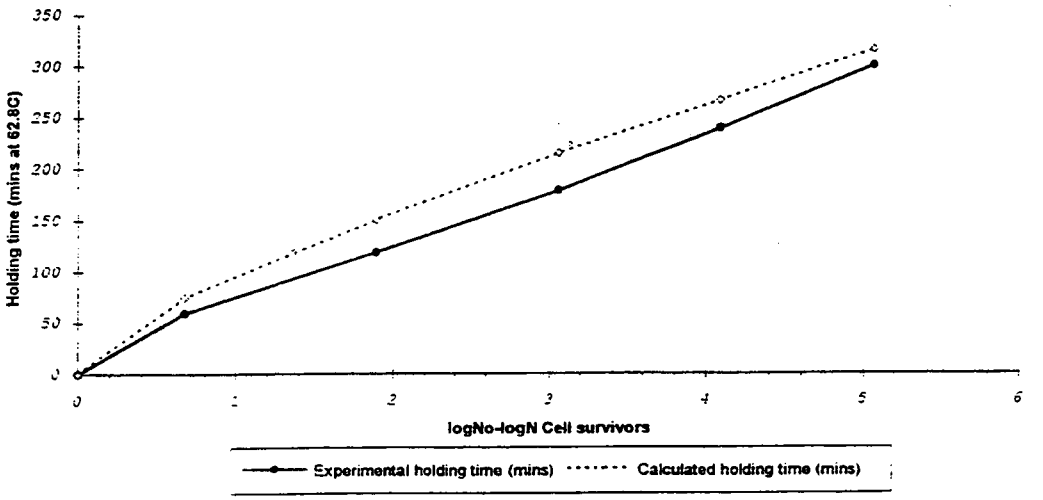


Figure 4.27 Calculated and experimental thermal death rate holding times for the same level of *Listeria* cell destruction at 62.8°C.

Moreover, once the k, c and a values have been established for the computation of the above ^{a}t formula, it was possible to elucidate a time for a fixed amount of cell destruction without having to refer to the thermal death rate curve. For example, the holding time at 62.8C required to reduce a cell population by 1.5 $\log_{10}$ cycles was shown to be 103.15 seconds.

$$^{a}t = \frac{(\log No - \log N)^{a}}{k} - \frac{c}{k} \quad = \quad \frac{(1.5)^{0.79}}{0.0119} - \frac{0.15}{0.0119} = 103.15 \text{ secs for a 1.5 log}$$

reduction in cell number at 62.8°C. The plot of ^{a}t against logNo-logN values would provide a calculated corrected thermal death curve to fit the experimental points.

When comparing different processes or microorganisms, a large number of log units of kill should be used so that the final calculation would incorporate the shoulder portion and the acceleration death rate phase of the cell survivor curve. For instance, under the present set of experimental heating conditions a 1 log unit destruction of *L monocytogenes* NCTC 11994 takes 98 secs, whereas the second unit takes 65 secs, the third unit takes 51 secs , the fourth unit takes 38 secs and the fifth unit takes only 29 secs (calculation from non-linearised survivor curve). Because of the shoulder on the

thermal death rate curve (Figure 4.27), more than half the total holding time (62.8°C) for a 5 log reduction was required for the first 2 log units of destruction. The need to observe a large number of log reduction units contrasted to the use of a single logarithmic D value for death rate calculations. The reciprocal of the thermal death rate constant k represents the holding time (mins or secs) for a 1 log cell reduction.

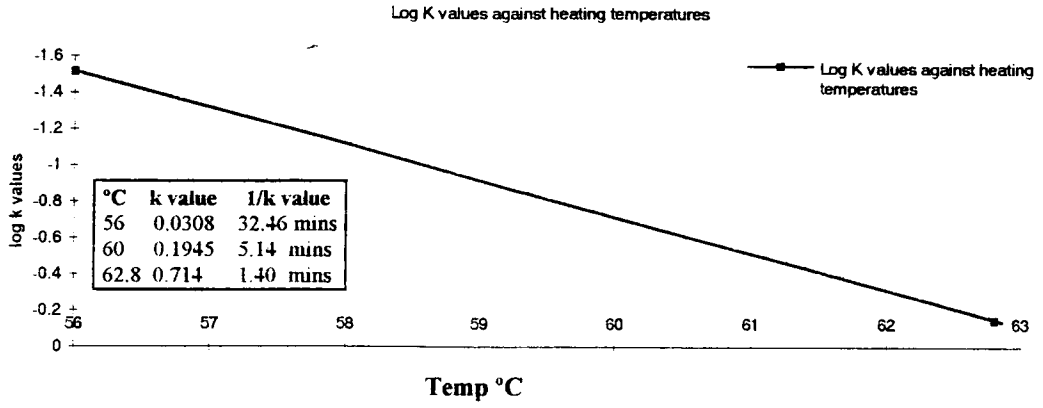
Other information was derived from the this linearised data, i.e. the k value was used as a reaction velocity constant to determine the temperature dependence of heat inactivation. Indeed, the log k values against temperature plot (Figure 4.28) was used like a Z plot to determine the k values for other heat inactivation temperatures. When the log of k values was plotted against their respective temperatures, the rate of cell destruction over the continuum of heat was calculated.

In Figure 4.28 only two heating temperature extremes (i.e.56°C and 62.8°C) were plotted against the respective log k values. Moreover, an approximate 1/k value for a certain lethal temperature can be determined by interpolating between the 2 heating temperatures for the unknown log k value. For example, the k value for the heat inactivation temperature of 60°C can be achieved by simply interpolating for the log k value at 60°C on the straight line plot. This log k value was shown to be 0.1945, by calculating the antilog and then the reciprocal of this thermal death rate constant k value, the holding time at 60°C for a 1 log reduction in cell number was estimated to be 5 mins 14 secs.

Having established that *L. monocytogenes* NCTC 11994 survivor curves could be linearised by application of the formula $(\log No - \log N)^a = kt + c$, attention now focused on applying this linearisation formula to the remainder to the thermal death rate curves produced by the 3 strains of *L. monocytogenes* under heating and holding at 56°C (for 60 mins) and/or 62.8°C (for 5 mins) in the previous thermal study (section 4.1.2.2.9).

The reciprocal of the thermal death rate constant k (for each strain of *L. monocytogenes* which had been cultured in SMA Gold Cap at 41°C and /or 37°C, heated at 56°C and /or 62.8°C and enumerated on TSYEA and/or LSA plating media) is illustrated in Table 4.13. A measure of the thermal resistance capability for each strain of *L. monocytogenes* was represented by the 1/k value, which is the holding

time (t mins) required to reduce a *Listeria* cell population (t_0 mins) by one log fold . Due to the large number of steps involved in the linearisation of a survivor curve, the individual stages involved in this linearisation were not shown and only the thermal death rate constant k and its reciprocal were illustrated.



°C	k value	1/k value
56	0.0308	32.46 mins
60	0.1945	5.14 mins
62.8	0.714	1.40 mins

Figure 4.28 Temperature dependence of death rate of *L. monocytogenes* NCTC 11994 cells used to interpolate and select k values.

As it was possible to linearise the log survivor curves, each combination of strain, cultivation temperature and heat inactivation temperature were assessed for their potential to increase *L. monocytogenes* heat resistance simply by comparing the times (1/k values) taken to reduce the cell concentration by this 1 log value.

Application of the non-parametric Wilcoxon Rank Sign test to the 1/k values in Tables 4.13 and 4.16 revealed that *L. monocytogenes* NCTC 11994 and 9863 cultivated at 41 °C and/or 37°C, heated at 56°C or 62.8°C and enumerated on TSYEA and/or LSA plates exhibited superior heat tolerance (P0.05) compared to strain NCTC 10357. The non-selective TSYEA plates provided greater recovery of heat damaged *Listeria* cells (P0.05) compared to stressed cells enumerated on LSA plates (i.e. for all 3 *L. monocytogenes* strains heat treated over a 60 minute period at 56°C and a 5 minute period at 62.8°C). Cultivation of *L. monocytogenes* at the elevated

temperature of 41°C significantly enhanced the heat resistance of all 3 strains enumerated on TSYEA and LSA plates, compared to the same cells grown at 37°C prior to heat treatment.

Table 4.13 Holding time (mins) required for a 1 log reduction in *Listeria* cell number as expressed by the reciprocal of the thermal death rate constant k value for cells which had been cultivated in SMA Gold Cap at 41°C, heated at 56°C and/or 62.8°C and enumerated on TSYEA and LSA plating media.

Holding Temp (°C)	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
60 mins at 56°C	32.46[†] (0.0308)[‡]	22.5 (0.044)	33.12 (0.0301)	24.2 (0.041)	6.27 (0.127)	3.54 (0.229)
5 mins at 62.8°C	1.4 (0.714)	1.08 (0.925)	1.36 (0.735)	1.18 (0.847)	0.48 (2.08)	0.36 ((2.77)

[†] Illustrates the reciprocal of the k value (i.e. the time in minutes, required to reduce a cell population by 1 log fold).
[‡] Illustrates the thermal death rate constant k derived from the linearised survivor curve (plot of (logNo-logN)^a =kt+c against holding time values).
[a,b,c] Are 3 strains of L. monocytogenes.

Therefore, it does appear that cultivation temperature, strain serotype (both NCTC 11994 and 9863 are designated as 4b while NCTC 10357 is of serotype 1a) did influence heat resistance capabilities when heated at 56°C and 62.8°C. The results from this linearisation study support the findings of the previous thermal study.

Table 4.14 Holding time (mins) required for a 1 log reduction in *Listeria* cell number as expressed by the reciprocal of the thermal death rate constant k value for cells which had been cultivated in SMA Gold Cap at 37°C, heated at 56°C and/or 62.8°C and enumerated on TSYEA and LSA plating media.

Holding Temp (°C)	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
60 mins at 56°C	10.61[†] (0.094)[‡]	5.85 (0.170)	10.45 (0.0956)	5.82 (0.172)	5.453 (0.1833)	ND[¶] (-)
5 mins at 62.8°C	0.96 (1.035)	0.78 (1.282)	1.02 (0.9804)	0.66 (1.515)	ND (-)	ND (-)

[†] Illustrates the reciprocal of the k value (i.e. the time in minutes, required to reduce a cell population by 1 log fold).

[‡] Illustrates the thermal death rate constant k derived from the linearised survivor curve [plot of (logNo-logN)^n=kt+c against holding time values].

[a,b,c] Are 3 strains of L. monocytogenes.

[¶] ND refers to failure to detect heat damaged L. monocytogenes during the holding period (To mins)

4.1.2.2.11 Determination of the heat resistance of *L. monocytogenes* in SMA White Cap (IMF).

The study in this section simply repeated the experimental procedure of section 4.1.2.2.10 using SMA White Cap, in order to confirm that a difference in thermal resistance existed between the 3 strain of *L. monocytogenes* and that this phenomenon was not soley attributed to a particular culture medium (i.e. SMA Gold Cap).

The results of this study illustrated the thermal death rate survivor curves by *L. monocytogenes cells* when cultivated in SMA White Cap prior to thermal inactivation at 56°C and 62.8°C. Indeed, Figures 4.29 and 4.30 revealed that these thermal death rate curves followed a similar pattern to those obtained in the previous heat inactivation study, where a shoulder section was observed at the earlier holding stages

preceded by an accelerated linear death rate. These figures represent heat treated cells which were enumerated on TSYEA plates.

In order to quantify the heat resistance potential of each strain it was necessary to apply the linearisation formula to straighten these survivor curves. Application of the linearisation formula $(\log No - \log N)^a = Kt + c$ successfully converted the thermal death rate curves to a straight line and Tables 4.15 and 4.16 show the reciprocal of the themal death rate constant k values (i.e. a measure of heat resistance).

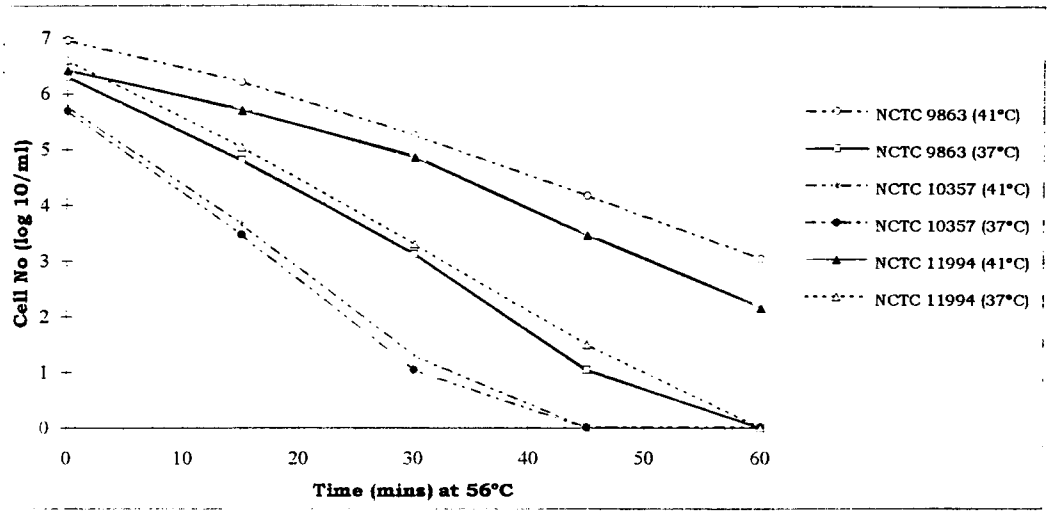


Figure 4.29 Thermal death rate curves for all three strains of *L. monocytogenes* which had been cultivated at 41°C and 37°C prior to heat treatment at 56°C.

Cultivation of cells in SMA White Cap (infant milk formula) resulted in significantly lower recovery of healthy and heat injured cells at the various holding times (at 56°C and/or 62.8°C) compared to growth in SMA Gold Cap prior to heat treatment (Figures 4.29 and 4.30). While the 1/k values were uniformly lower compared to the previous heating experiment (SMA Gold Cap), a similar trend in the recovery of heat treated cells emerged as related to cultivation temperature, strain serotype and enumeration plating media. *L. monocytogenes* strains NCTC 11994 and 9863 were significantly more heat tolerant, at both bactericidal temperatures (56°C and 62.8°C),

compared to strain 10357 cultivated, heated and enumerated under the same conditions. All strains of *L. monocytogenes* cultivated at 41°C prior to heat treatment were significantly more heat tolerant (P0.05) compared to the same strains grown at 37°C (Table 4.15 and Table 4.16).

Pleomorphic surface colonies of *L. monocytogenes* emerged on TSYEA and LSA plates at both heat inactivation temperatures. The variation in colony form was marked by irregularities in the outer margin or colony edge. A Gram stain prepared from this section showed that the cells were arranged singly, paired and in long chains (a filamentous appearance). The non-heat treated test cultures of *L. monocytogenes* failed to support the development of any R-form *Listeria* colonies.

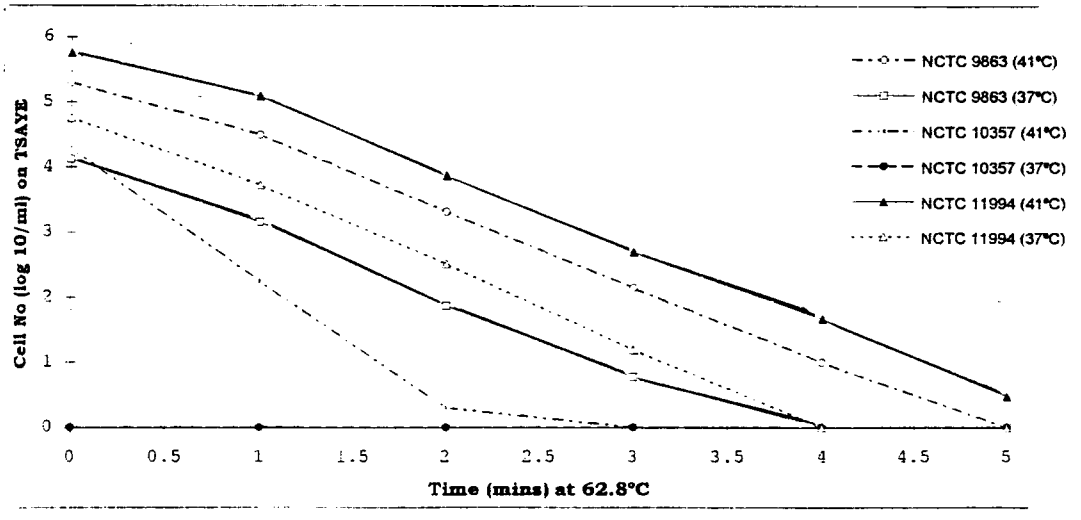


Figure 4.30 Thermal death rate curves for all three strains of *L. monocytogenes* which had been cultivated at 41°C and 37°C prior to heat treatment at 62.8°C.

Table 4.15 Holding time (mins) required for a 1 log reduction in *Listeria* cell number (expressed as the reciprocal of the thermal death rate constant k value obtained from a linearised survivor curve) which had been cultivated in SMA White Cap at 41°C, heated at 56°C and/or 62.8°C and enumerated on TSYEA and LSA plating media.

Holding Temp (°C)	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
60 mins at 56°C	15.83[†] (0.0632)[‡]	11.75 (0.0851)	17.11 (0.058)	16.52 (0.0605)	7.92 (0.1262)	4.58 (0.2183)
5 mins at 62.8°C	1.33 (0.751)	0.805 (1.242)	1.28 (0.781)	1.075 (0.93)	0.56 (1.785)	0.46 (2.1739)

[†] Illustrates the reciprocal of the k value (i.e. the time in minutes, required to reduce a cell population by 1 log fold).
[‡] Illustrates the thermal death rate constant k derived from the linearised survivor curve [plot of $(logNo-logN)^a = kt+c$ against holding time values].
a,b.c Are 3 strains of *L. monocytogenes*.

Table 4.16 Holding time (mins) required for a 1 log reduction in *Listeria* cell number (expressed as the reciprocal of the thermal death rate constant k value obtained from a linearised survivor curve) which had been cultivated in SMA White Cap at 37°C, heated at 56°C and/or 62.8°C and enumerated on TSYEA and LSA plating media.

Holding Temp (°C)	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
60 mins at 56°C	9.54 (0.104)[‡]	5.65 (0.176)	9.70 (0.103)	5.72 (0.174)	6.02 (0.106)	3.25 (0.307)
5 mins at 62.8°C	0.95 (1.052)	0.76 (1.31)	1.06 (0.943)	0.89 (1.12)	ND (-)	ND (-)

[†] Illustrates the reciprocal of the k value (i.e. the time in minutes, required to reduce a cell population by 1 log fold).
[‡] Illustrates the thermal death rate constant k derived from the linearised survivor curve [plot of $(logNo-logN)^a = kt+c$ against holding time values].
a,b.c Are 3 strains of *L. monocytogenes*.

4.1.2.2.12 Determination of the heat resistance of 3 strains of *L. monocytogenes* in a variety of culture media at 3 heat inactivation temperatures (56°C, 60°C, and 62.8°C).

The experiential design and results produced from the previous 2 thermal experiments have revealed that whether or not *L. monocytogenes* was cultivated at 41°C or 37°C prior to heat treatment at 56°C or 62.8°C, the resulting thermal death rate plot of $\log_{10}$ cell number of survivors against holding time (mins) consistently fitted a survivor curve rather than a logarithmic straight line. Furthermore, it has been revealed that all three strains of *L. monocytogenes* cultivated at the elevated temperature of 41°C exhibited superior thermotolerance compared to the same *Listeria* cells grown at 37°C prior the thermal inactivation at either 56°C or 62.8°C. Under the present set of experimental conditions, pleomorphic colony forms of *L. monocytogenes* have emerged and their identity confirmed. The majority of these R-form surface cultures were recovered on the non-selective TSYEA plates which had been initially cultivated at 41°C (as compared to 37°C) prior to heat treatment.

This is the first in a series of 4 thermal experiments to investigate the effect of media composition on both the heat resistance of *L. monocytogenes* and the ability of certain culture media to support the emergence of atypical colonial variants of this organism. Furthermore, the 3 strains were heat treated at 60°C in addition to the holding of cells at the lethal temperatures of 56°C and/or 62.8°C, this extra holding temperature of 60°C should provide a more precise determination of the rate of cell death against a contimuum of bactericidal temperatures.

Therefore, as each strain of a particular bacterium may exhibit a different response to heating at different temperatures, this particular amount of temperature increase (i.e. required to reduce the log 1/k values 1 log fold) is characteristic of the heat resistance of that particular strain and may be used to establish and/or monitor the thermotolerance of an organism under any a variety of culture conditions.

Interpolation of log k against holding temperature values in a previous thermal study (Figure 4.28), revealed a 1/k value of approximately 6 to 7 mins for strains NCTC

11994 and NCTC 9863 at 60°C and on the basis of this information a 5 to 6 log fold reduction should occur in approximately 30 mins. However, the plot of log k value against time for the heat sensitive strain NCTC 10357 resulted in a prediction of 1 to 2 mins for the 1/k value at the same holding temperature of 60°C (this implies a holding period range of 0 to 10 mins for this commensurate 5 to 6 log reduction in *Listeria* cell concentration).

Furthermore, it was deemed necessary to modify the sampling times for strain NCTC 10357 heated at 62.8°C, as the range of holding times was too severe to construct a proper thermal death rate plot (especially for *Listeria* cells cultivated at 37°C prior to heat treatment, as heat treated cells failed to recover on both types of plating media over the holding period at 62.8°C).

This study was initiated by subculturing *L. monocytogenes* strains NCTC 11994, 9863 and 10357 in separate BHI broths prior to inoculation into either tyndallised SMA Gold Cap, Farleys First Milk, Whole milk or TSYEB. Due to the enormity of this experimental protocol, this thermal investigation was sectioned into 4 studies, i.e. a separate thermal study for each of the 4 different culture media was conducted. The experimental protocol was the same as that employed in section, and for practical purposes the results from the latter 3 culture media were presented in a tabulated form at the end of this study.

The 3 test SMA Gold Cap cultures were cultivated at 41°C only (in a stationary position) for 24 hours prior to heat treatment at 56°C, 60°C and 62.8°C. Only one cultivation temperature was employed due to the large number of test strains and the incorporation of an additional heat inactivation temperature. Each test culture was brought from room temperature to the equilibrated temperature of each heat inactivation temperature (a warming up period was allowed for in the calculation of the actual holding periods at the respective heating temperatures). The SMA Gold Cap was employed as the cultivation, heating menstruum and diluent as described in the previous thermal experiments (section 4.1.2.2.9).

The thermal death rate plots of $\log_{10}$ number of cell survivors against holding times for all three strains of *L. monocytogenes* heated at the various holding temperatures in SMA Gold Cap exhibited thermal death curves similar to the previous heating

experiments. The range of holding times employed at each respective lethal temperature was adequate in reducing the initial cell population by several log fold. Figures 4.31, 4.32 and 4.33 showed the thermal death rate curves of all 3 strains of *L. monocytogenes* which had been heat treated at 56°C, 60°C and 62.8°C respectively (prior to enumeration on TSYEA plates).

Survivor curves for heat stressed cells which had been enumerated on the selective plating medium LSA were not illustrated as it was felt that the general pattern of cell survival was similar to that of TSYEA plates. Indeed, the sample time intervals at the holding temperature of 60°C was approximately equal to the holding time required to obtain a 1 log cell number reduction (1/k value) as predicted from interpolation of previous log K versus holding temperature values.

It has been illustrated in Table 4.17 that the thermal death rate constants for all three strains had followed a similar pattern compared to the previous heating experiments where *Listeria* cells from NCTC 11994 and 9863 were more heat tolerant (compared to NCTC 10357). Greater resuscitation of thermally stressed cells occurred on the non selective TSYEA plating medium.

Although certain plates exhibited a high conversion rate (> 20%) to the R- form colony type post heat treatment, generally this conversion rate was quite low for serotype 4b strains of *L. monocytogenes* (mean for TSYEA and LSA was 3.4% and 2.5% respectively) on plates shown to permit pleomorphic development. However, the frequency of conversion appeared higher for the two serotype 4b strains (i.e. NCTC 11994 and 9863) compared to that of NCTC 10357 (this less heat resistant strain had a conversion rate of 1.6% and 1.2% on TSYEA and LSA plates respectively).

Table 4.17 Holding time (mins) required for a 1 log reduction in cell number (expressed as the reciprocal of the thermal death rate constant k value obtained from a linearised survivor curve) which had been cultivated in SMA Gold Cap at 41°C, heated at 56°C,60°C and/or 62.8°C and enumerated on TSYEA and LSA plating media.

Holding Temp (°C)	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
56	30.05† (0.033)‡	21.26 (0.047)	30.33 (0.0329)	22.21 (0.045)	7.54 (0.1326)	4.26 (0.235)
60	5.88 (0.170)	4.41 (0.226)	5.22 (0.191)	4.55 (0.219)	1.42 (0.704)	1.36 (0.735)
62.8	1.25 (0.8)	1.02 (0.98)	1.31 (0.763)	1.06 (0.943)	0.479 (2.087)	0.34 (2.941)

† Illustrates the reciprocal of the k value (i.e. the time in minutes, required to reduce a cell population by 1 log fold).
‡ Illustrates the thermal death rate constant k derived from the linearised survivor curve [plot of $(\log No - \log N)^a = kt+c$ against holding time values].
a,b,c Are 3 strains of L. monocytogenes.

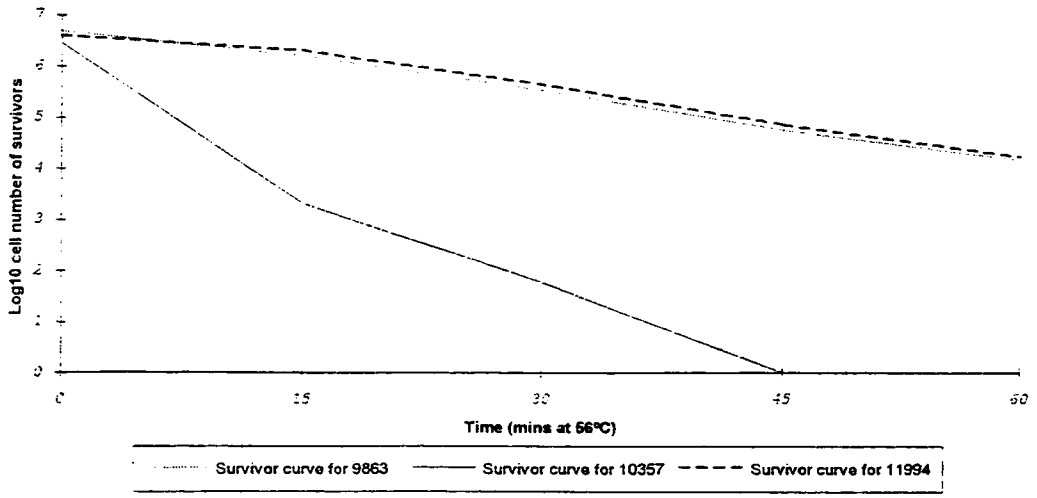


Figure 4.31 The thermal death rate curves of *L. monocytogenes* NCTC 11994 , NCTC 9863 and NCTC 10357 which had been cultivated at 41°C prior to heat treatment at 56°C.

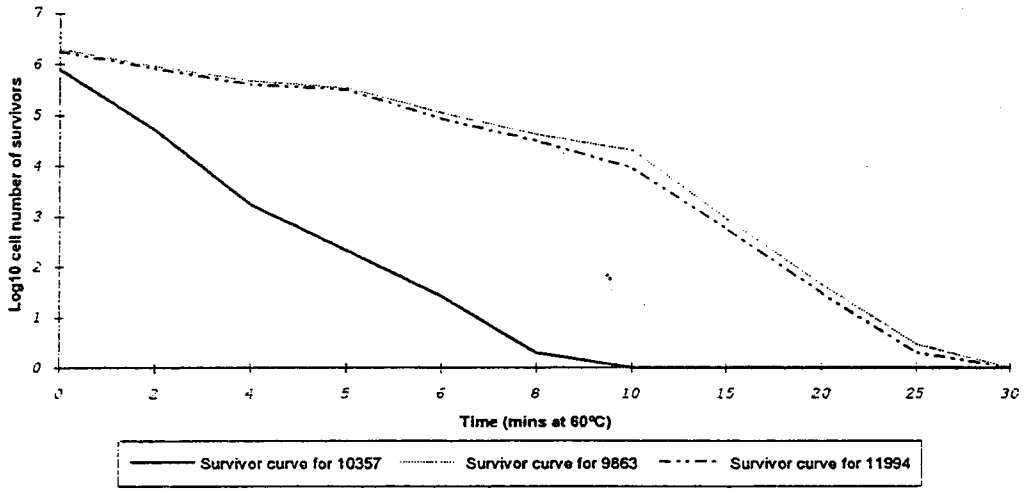


Figure 4.32 The thermal death rate curves of *L. monocytogenes* NCTC 11994 , NCTC 9863 and NCTC 10357 which had been cultivated at 41°C prior to heat treatment at 60°C.

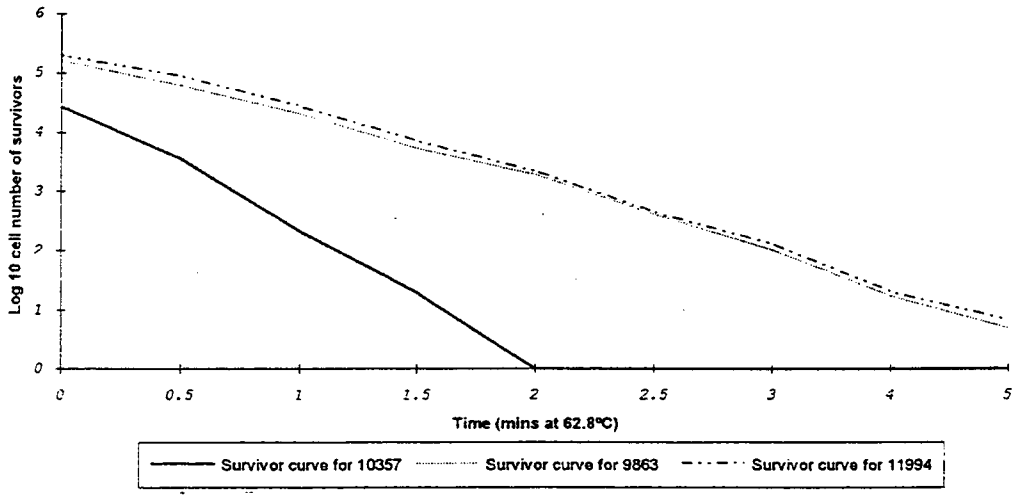


Figure 4.33 The thermal death rate curves of *L. monocytogenes* NCTC 11994 , NCTC 9863 and NCTC 10357 which had been cultivated at 41°C prior to heat treatment at 62.8°C.

Indeed, the plot of $\log_{10}$ cell number of survivors against holding temperature for the 3 strains cultivated and heated in tyndallised Farleys First Milk, Whole milk and TSYEB at 56°C, 60°C and/or 62.8°C resulted in all these strains exhibiting thermal death rate curves, which were characterised by an initial shoulder portion preceding an accelerating thermal death rate (Figures 4a to 12a in appendix). These survivor curves were evident for all three strains of *L. monocytogenes* which had been heated at the above temperatures for various holding times in all the test culture media. Linearisation of all these thermal death rate curves was achieved by application of the formula $(\log No - \log N)^a = Kt + c$. Computation of the reciprocal thermal death rate constant k (which is a measure of the heat resistance capability of each strain heat at the above temperatures) preceded each linearisation procedure. This 1/k value is an approximation as to the holding time required to reduce the initial cell population (at T0 mins) by one log fold. The 1/k values for all strains of *L. monocytogenes*, cultivated in the 4 test media and heated at 56°C, 60°C and 62.8°C are illustrated in Table 4.18.

While heat treated cells belonging to the serotype 4 b strains (i.e. NCTC 11994 and 9863) were recovered in greater numbers at both cultivation temperatures, at each heat inactivation temperature, in all culture media and on both plating media compared to *L. monocytogenes* NCTC 10357 (P0.05), the greatest recovery of heat subjected cells occurred for all strains cultivated at 41°C prior to heating. A superior recovery of heat treated *Listeria* cells was achieved when the samples were plated onto a non-selective TSEYA plating medium. The serotype 4b strains did not exhibit any significant difference between the level of stressed cell recovery at each heat inactivation temperature, in all culture and plating media. While SMA Gold Cap consistently supported high recoveries of uninjured and sublethally stressed cells belonging to serotype 4b, this effect was shown to be not significant (P0.05). Media composition did not alter the thermotolerance capabilities of NCTC 10357 (serotype 1a) at any holding temperature (P0.05).

Table 4.18 illustrates the 1/k values {equivalent to holding time (mins) required to reduce a *Listeria* cell population by 1 log fold} for the 3 strains of *L. monocytogenes* which had been cultivated in a variety of culture media at 41°C prior to heat treatment at 56°C, 60°C and 62.8°C.

Holding Temp (°C)	Test Medium	NCTC 11994[a]		NCTC 9863[b]		NCTC 10357[c]	
		TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
56	SMA Gold Cap	30.05[†]	21.26[‡]	30.33	22.21	7.54	4.26
	Farleys First Milk	25.57	19.41	26.51	20.04	7.74	4.27
	Whole Milk	23.15	18.16	24.31	20.59	7.21	3.89
	TSYEB	30.9	23.6	59.4	25.4	6.51	4.22
	(mean)	**24.42**	**20.6**	**27.64**	**22.06**	**7.25**	**4.16**
60	SMA Gold Cap	5.88	4.41	5.22	4.55	1.42	1.36
	Farleys First Milk	5.79	5.13	5.65	4.96	1.37	1.19
	Whole Milk	5.91	4.55	6.11	4.75	1.51	1.33
	TSYEB	5.16	4.16	5.12	4.08	1.30	1.15
	(mean)	**5.69**	**4.56**	**5.53**	**4.59**	**1.40**	**1.26**
62.8	SMA Gold Cap	1.25	1.02	1.31	1.06	0.48	0.34
	Farleys First Milk	0.93	0.84	0.95	0.91	0.49	0.34
	Whole Milk	0.93	0.8	1.09	0.84	0.47	0.4
	TSYEB	1.15	0.91	1.11	0.89	0.45	0.38
	(mean)	**1.07**	**0.89**	**1.12**	**0.93**	**0.45**	**0.37**

[a.b.c] strains of *L. monocytogenes*
[†,‡] 1/k values (minutes at 56, 60 and 62.8°C)

The 1/k values illustrated in Table 4.18 confirm the above findings, where serotype 4b *Listeria* strains (compared to serotype 1a); cultivation at 41°C prior to heating (these properties influencing thermal resistance); and recovery on TSYEA plates (as opposed to LSA plates) resulted in greater decimal reduction times exhibited at each heat inactivation temperature (P 0.05). An increase in heat treatment temperature corresponded to a decrease in 1/k value (analagous to logarithmic decimal reduction times, in minutes) required to produce the same level of cell destruction.

Where a change in morphological form occurred, the frequency of conversion from the S to the R-form for NCTC 11994 and 9863 was moderate on TSYEA (mean 3.5% and 2.57%) and LSA plates (mean 3.0% and 1.2%) respectively, while strain NCTC 10357 appeared quite stable in colony form exhibiting less than 1% and 0% conversion on TSYEA and LSA plates respectively. It must be noted that the above conversion percentages relate to a plate count of $\geq$ 200 CFU ml^{-1}. Generally stability varied from 0 to 86% on subculturing and no particular combination of heat inactivation temperature and/or strain type appeared to improve this stability factor (stability appeared exclusive to the strain itself and was not governed by environmental influences. The rough colony forms were characterised by variation in opacity, elevation and the appearance of the outer colony margin compared to that of standardised S-forms.

Rough surface colonies of *L. monocytogenes* were recovered from heat treated whole milk. However, *Listeria* strain NCTC 10357 failed to produce any surface culture variants under the test conditions and a reduced frequency of S-form conversion was observed by serotype 4 b strains. The actual frequency of conversion for *L. monocytogenes* NCTC 11994, 9863 and 10357 to R-form colonies (as detected by TSYEA plates) was 1.8%, 2.25% and 0.81% respectively. Whereas the S-form conversion rate were 1.22%, 1.56% and 0% (as detected by LSA plates) for the same strains respectively. It would appear LSA is an inferior medium for the detection of R-form *Listeria* colonies under the present set of heating and recovery conditions. Furthermore, the stability of R-form type varied significantly (P0.05) with a range of 3.5 to 92%.

The emergence of pleomorphic surface cultures in these milk products may be due to the combined influence of stationary cultivation at elevated temperatures and heat stressing by exposure to sublethal heating conditions. It was previously demonstrated (4.1.2.2.7) that *Listeria* cells cultured at 37°C prior to heat inactivation did convert to the R-form. However, the generation of R-form *L. mononcytogenes* colonies was infrequent (< 1% where conditions were advantageous for pleomorphic colony development) compared to the same strains which had been initially cultivated at 41°C prior to heat treatment.

TSYEB was revealed to be a poor medium for the detection of atypical colonies of *L. monocytogenes* as indicated by the lowest conversion rate to the R-form. Indeed, S-form serotype 4b colonies varied in colony morphology (post heat treatment) at a frequency of <1.0% on both plating media. No pleomorphic surface cultures were observed on any plates which were seeded with heat stressed *Listeria* cells previously grown at 37°C. Non heat treated test cultures of *L. monocytogenes* failed to produce any R-form colonies. Less than 1% of the heat treated *L. monocytogenes* NCTC 10357 cells were converted to R-forms on TSYEA plates and no surface variants emerged on LSA.

This thermal inactivation study demonstrated that R-form colonies of *L. monocytogenes* do exist and that they can be reproducibly generated under controlled experimental conditions. Table 4.18 was constructed in order to illustrate the effects of variation in test media composition (if any) on the subsequent thermotolerance of three S-form types of *L. monocytogenes* (NCTC 11994, 9863 and 10357). Furthermore, it has been established that differences in *L. monocytogenes* serotype, cultivation temperature prior to heat treatment and enumeration medium may influence the recovery of heat stressed cells of *L monocytogenes* exposed to bactericidal temperatures.

4.1.2.2.13 The heat resistance characteristics of rough and smooth forms of *L. monocytogenes*.

Having established that a difference in recovery of healthy and heat injured cells occurs between strains of *L. monocytogenes* and that this variation may be attributed to various inter-related cultural parameters including the serotype employed, the objective of this study was to investigate whether a difference in the heat resistance exists between various colony forms of this organism. Having investigated the inherent heat resistance abilities of both colony forms and elucidated an optimum set of recovery conditions for future implementation, it should then be possible to address the problem of recovering and detecting heat damaged and/or healthy *L. monocytogenes* cells which may exist in contaminated infant milk formulae.

It is of paramount importance that one is able to tentatively quantify the thermotolerance potential of all *L. monocytogenes* forms; to arrive at an optimum set of recovery conditions for heat treated cells and to know if *L. monocytogenes* can vary in morphological form in order to confidently address the issue of detecting small numbers of uninjured or heat treated *L. monocytogenes* among the microbial flora in infant milk formula.

This is the first in a series of thermal treatments where R and S-form colony types of *L. monocytogenes* were compared for heat resistance ability under the same set of defined heating conditions. As it has been demonstrated that the 2 serotype 4b S-forms of *L. monocytogenes* (i.e. NCTC 11994 and 9863) share similar thermotolerance abilities, it was therefore decided to just employ NCTC 11994 as the standard smooth form. A surface culture variant of NCTC 9863 (i.e. NRB2) exhibiting 96% stability was selected as the test R-form colony for this heat study. Stability was determined by cultivating an isolated R-form colony in TSYEB to a concentration of approximately 10^8 CFU ml^{-1}, spread plating the 10^{-6} dilution and enumerating the number of R-form conversions on TSYEA and LSA plates. In order to standardise the cell concentration through all stages of heating it was necessary to use a non-dairy test medium (e.g. TSYEB).Isolated colonies of both morphological forms (NCTC 11994 and NRB2) were obtained on TSYEA by the streak plate method (i.e.

The cultivating, heating, diluting and enumerating conditions employed in this study were similar to the previous thermal studies. However, the test cultures were

cultivated in TSYEB at either at 37°C (110 rpm) and/or 41°C (0 rpm) prior to heat treatment at 56°C and 60°C. was carried out prior to inoculation of test culture media. The cell concentration of the 24 hour adjusted S-form and R form subcultures was shown to be $1.64x10^9$ and $9.7x10^8$ CFU ml^{-1} respectively.

The thermal death rate plots of log_{10} cell number of survivors against holding time values are illustrated on Figures 4.34 and 4.35. A thermal death rate survivor curve was observed for the S and R-form heat treated test cultures at both holding temperatures. It was evident that a similar survivor curve trend emerged for both morphological colony forms, this maybe due to NRB2 being a direct descendant of NCTC 9863 (which has a similar thermotolerance to NCTC 11994). However, while both forms exhibited a shoulder portion preceding a linear thermal death rate, it did appear that this R-form also exhibits a tailing effect at the longer holding times.

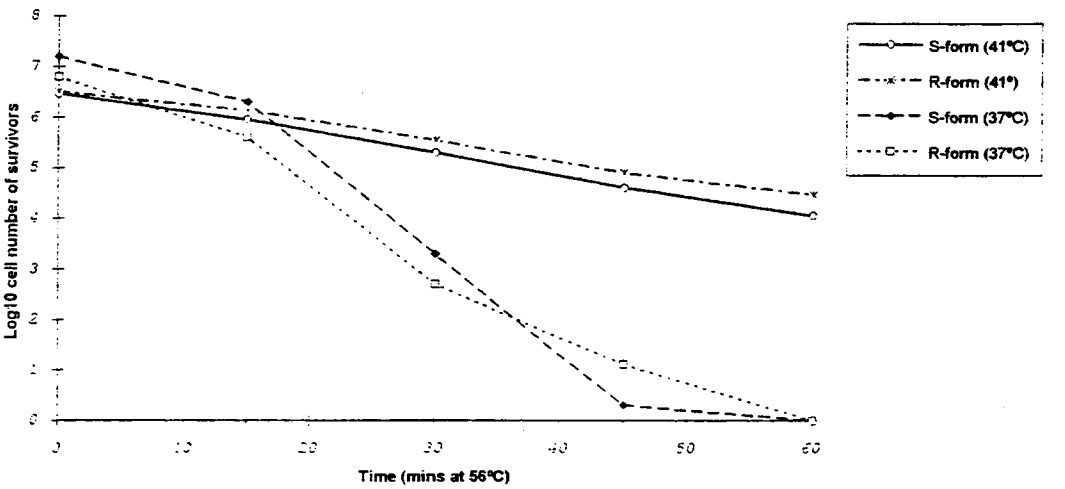


Figure 4.34 Thermal death rate curves for the S-form (NCTC 11994) and R-form (NRB2) *L. monocytogenes* which had been cultivated at 41°C and 37°C prior to heat inactivation at 56°C and enumeration on TSYEA plates.

This apparent tailing portion exhibited by NRB2 had not been demonstrated by the S-form in previous thermal inactivation studies. The question now arose as to the ability

of the linearisation formula $(logNo-logN)^a = kt+c$ to straighten a survivor curve which possesses a shoulder and a tail section. Failure to linearise both types of survivor curve would make it extremely difficult to compare the heat resistance potential of both colony forms (as expressed in terms of 1/k values) at each heat inactivation temperature.

Application of the linearisation formula to the NRB2 thermal death rate curve constructed from log_{10} cell survivors against holding time values at 60°C did not straighten the survivor curve. Moreover, the subsequent plot of log(logNo-logN) against log time values did not conform to a straight line plot (Figure 4.36). Therefore computation of the *a* value (i.e. the reciprocal of the slope) was not possible, resulting in an inability to linearise this R-form survivor curve. Indeed, the plot of R-form Log(logNo-logN) against log time values at a heating temperature of 56°C confirmed that a straight line plot was not possible, thus an *a* value could not be determined (Figure 4.37). The calculation of an accurate *a* value was critical in order that the formula $(logNo-logN)^a = kt+c$ could be employed to linearise survivor curves.

The formula $(logNo-logN)^a = kt+c$ was not suitable for linearisation of R-form colonies of *L. monocytogenes* which exhibited both a shoulder and tail section in the survivor curve. Failure to obtain a suitable *a* value at 56°C resulted in an inability to straighten the R-form thermal death rate curve. All the thermal death rate curves exhibited by the S-form were successfully linearised by application of the above formula. Figure 4.38 proves that the S-form survivor curves could be successfully linearised (where NCTC 11994 was cultivated at 41°C prior to heating at 56°C).

The 1/k values could not be calculated for NRB2 which made comparison of heat resistance abilities between the two morphological forms difficult. However, considering that both *Listeria* forms had a similar cell concentration prior to heat treatment and by virtue of the fact that heat stressed cells of the R-form were recovered at longer holding times (compared to the S-form), this would imply that the rough form was more heat tolerant. In order to tentatively show the variation in heat resistance between colony forms, a best fit straight line was drawn between the data points of log_{10} cell number against holding times and the logarithmic decimal

reduction time (D value) calculated. Where possible the equivalent 1/ k values (mins) were also illustrated (Table 4.19).

From Table 4.19 it was apparent that the calculation of a logarithmic D value from a best fit straight line (which was constructed through the thermal death rate curved data points) was in fact a poor estimate of the true thermotolerance capability of *L. monocytogenes.* The 1/k values derived from the linearised S-form survivor curves gave a more accurate representation of the time required to reduce a cell population by one log fold as it took into consideration the shoulder section as well as the curved accelerating death rate. Therefore, consistently longer decimal reduction time values (i.e. 1/k values) were observed for the same log reduction in cell number compared to the corresponding logarithmic D value at each holding temperature.

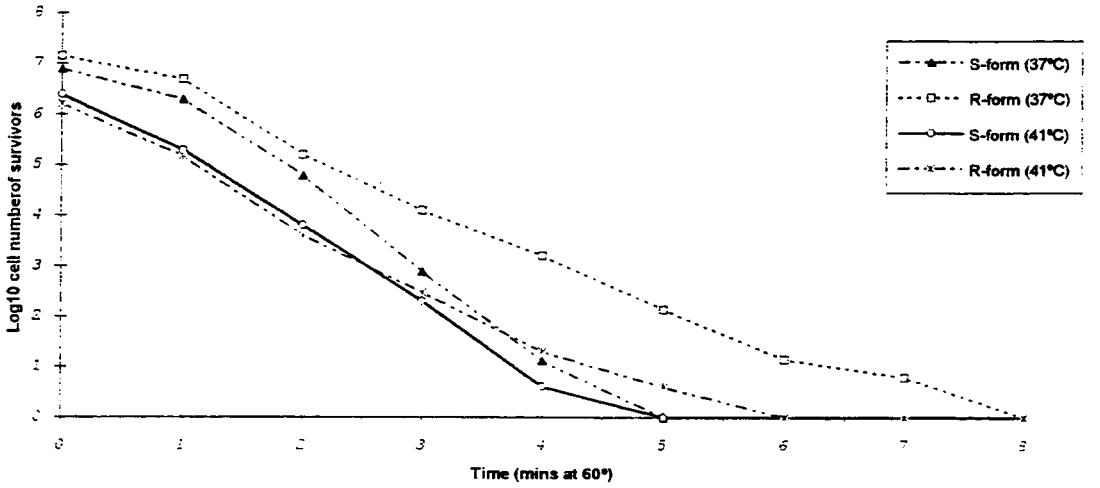


Figure 4.35 Thermal death rate curves for the S-form (NCTC 11994) and R-form (NRB2) *L. monocytogenes* which had been cultivated at 41°C and 37°C prior to heat inactivation at 60°C and enumeration on TSYEA plates.

From an observation of the thermal death rate curves (Figures 4.37) and the summary of heat resistance abilities (as expressed in D and 1/k values in Table 4.26), it would appear that the R- colony form (NRB2) of *L. monocytogenes* may be more heat resistant than the S-form (NCTC 11994) under study.

Furthermore, Table 4.19 illustrated that a far greater heat resistance was achieved (P 0.05) by the 2 *Listeria* colony forms when the cells were initially cultivated 41°C in a stationary position prior to heat treatment (as opposed to 37°C under orbital cultivation). The plot of log 1/k values against respective heat inactivation temperatures was not performed, as accurate k values were not available and only 2 heat treatment temperatures were employed.

As the tailing effect exhibited by NRB2's thermal death rate survivor curve is cause for concern, the study was repeated using a different Rough form of L. monocytogenes (NRB5).

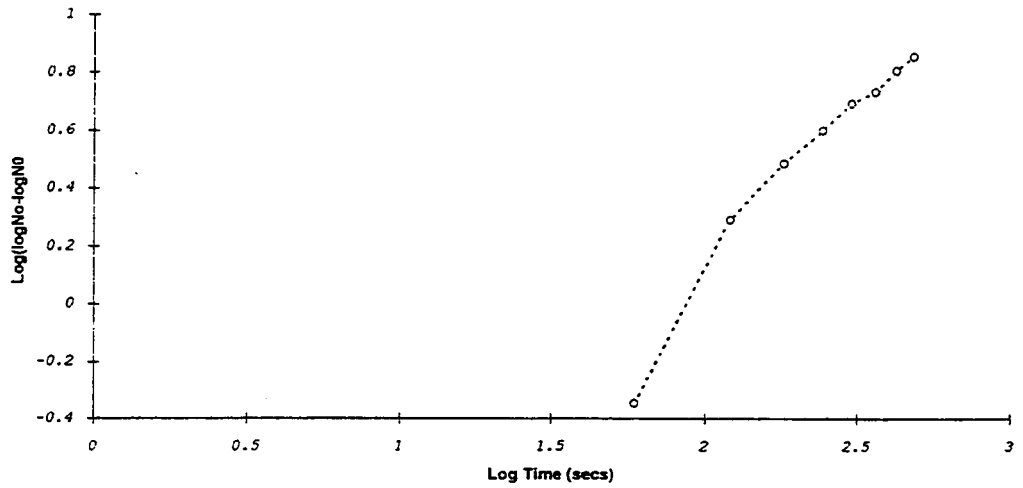


Figure 4.36 illustrates a non linear plot of log(logNo-logN) against log time values for NRB2 previously cultivated at 37°C prior to heating at 60°C, making it impossible to calculate an *a* value.

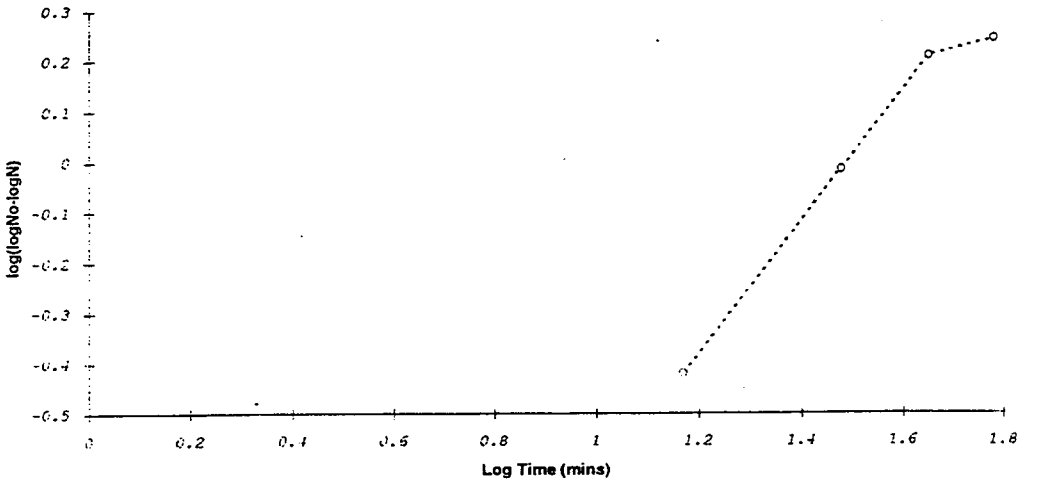


Figure 4.37 illustrates a non linear plot of log(logNo-logN) against log time values for NRB2 previously cultivated at 41°C prior to heating at 56°C, making it impossible to calculate an *a* value.

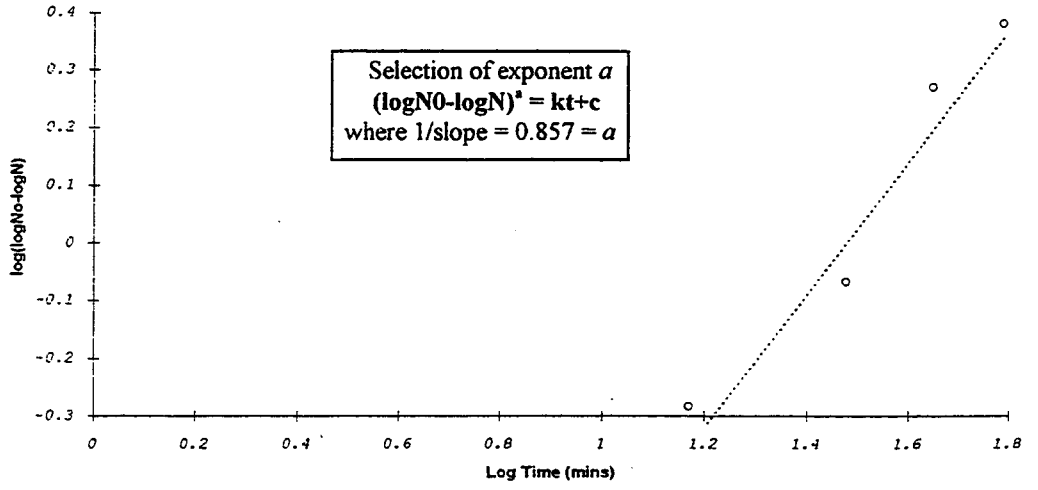


Figure 4.39 illustrates a linear plot of the log(logNo-logN) against log time values for the S-form heat stressed cells of NCTC 11994 which were previously cultivated at 41°C prior to heat treatment at 56°C.

Table 4.19 Approximate D values for S and R-colony forms of *L. monocytogenes*, calculated by plotting a best fit straight line through the curved data points of Log_{10} number of cell survivors against holding time at either 56°C or 60°C.

Colony form *L. monocytogenes*	D and 1/k value at 56°C[c]		D and 1/k value at 60°C[d]	
	37°C (110 rpm)[a]	41°C (0 rpm)[b]	37°C (110 rpm)	41°C (0 rpm)
S-form (NCTC 11994)	6.42‡ (13.69)†	24.89 (30.05)	0.657 (1.27)	3.455 (3.70)
R-form (NRB5)	7.908	32.48	1.057	4.909

‡ Estimated D value in minutes at 56°C holding
† 1/k value in minutes at 56°C
[a,b] Cultivation conditions prior to heat treatent at 56°C
[c,d] Heat treatment temperature

It must be stressed that while one R-form colony type showed a shoulder and tail section in its survivor curve, it does not necessarily mean that all R-forms possess this tailing ability. However, demonstration of tailing by a limited number of R-form colony types should be sufficient to cause concern.

4.1.2.2.14 Confirmation that R-form *Listeria* colonies exhibit a shoulder and tailing effect in their thermal death rate curves.

This thermal study simply repeated the previous experimental protocol using NRB5 as the R-form test culture. Two S-form surface cultures (NCTC 11994 and 9863) were incorporated into the heating study in order to compare the heat resistance abilities of each colony form under the same set of cultivation, heat inactivation and enumeration conditions. The test cultures were held at 56°C and 60°C for the time intervals mentioned in the previous thermal study. The 24 hour test cultures were not spectrophotometrically (A_{440nm}) adjusted prior to heat treatment, therefore the cell concentration for all strains cultivated at 41°C (0 rpm) and 37 (110 rpm) were shown to be approximately 10^8 and 10^9 CFU ml^{-1} respectively (which was confirmed by total aerobic mesophilic counts).

Results from this study revealed a shoulder and tail section in the R-forms (NRB5) thermal death rate curve (Figure 4.39, 4.40 and 4.41). These figures illustrate the thermal death rates for both the smooth and rough culture forms of *L. monocytogenes* which were cultivated at 41°C (Figure 4.39) and 37°C (Figure 4.40) prior to heat treatment at 60°C. Linearisation of these non-logarithmic survivor curves was not possible viz. the formula (logNo-logN)a = kt+c. The 2 S-form colony types (serotype 4b) exhibited a similar heat resistance ability under the test conditions, which was inferior to the thermotolerance of NRB5. The smooth colony types exhibited a shoulder section only in the thermal death rate plot of $\log_{10}$ cell number of survivors against holding times. Similar thermal death rate curves were exhibited when both morphological forms were heat treated at 56°C (Figure 4.41).

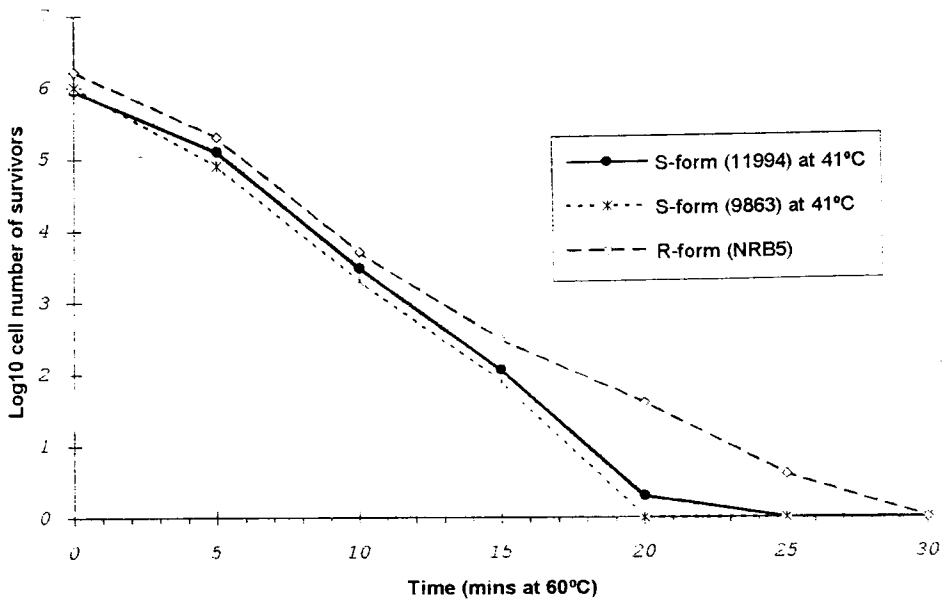


Figure 4.39 Thermal death rate curves for the S-form (NCTC 11994 and 9863) and R-form (NRB5) *L. monocytogenes* which had been cultivated at 41°C (0rpm) prior to heat inactivation at 60°C and enumeration on TSYEA plates.

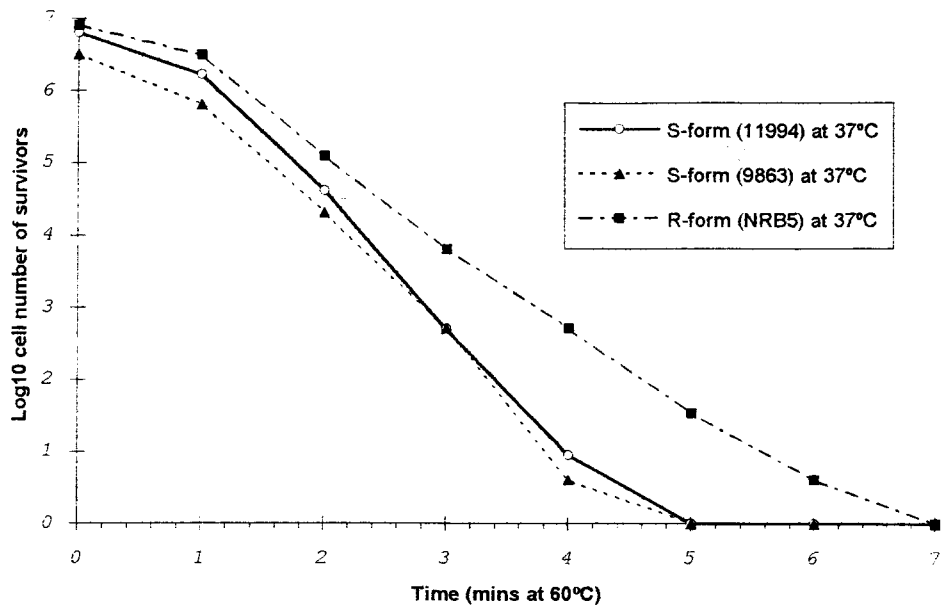


Figure 4.40 Thermal death rate curves for the S-form (NCTC 11994 and 9863) and R-form (NRB5) *L. monocytogenes* which had been cultivated at 37°C (110 rpm) prior to heat inactivation at 60°C and enumeration on TSYEA plates.

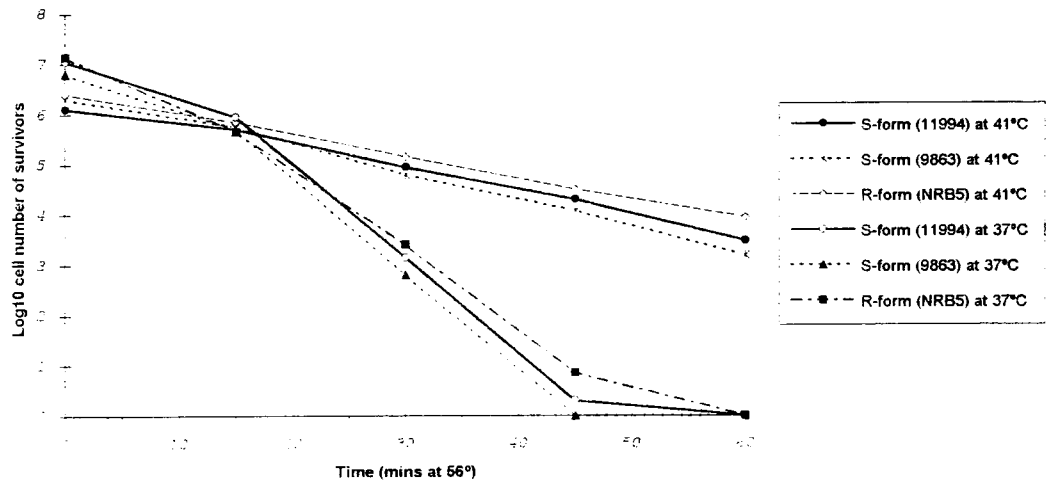


Figure 4.41 Thermal death rate curves for the S-form (NCTC 11994 and 9863) and R-form (NRB5) *L. monocytogenes* which had been cultivated at 41°C (0rpm) or 37°C (110 rpm) prior to heat inactivation at 56°C and enumeration on TSYEA plates.

Listeria cells which were shaken at 110 rpm during cultivation at 37°C reached a 1 log fold higher cell concentration (10^9 CFU ml^{-1}) compared to cells that were statically cultivated (10^8 CFU ml^{-1}). This may explain why the concentration of cells at T_0 mins (i.e. terminus of warming up period) was greater for cells cultivated at 37°C (110 rpm) prior to heat treatment.

It was evident from the thermal death rate curves plotted above that NRB5 exhibited a tail section in its survivor curve. As linearisation of this type of survivor curve is not possible, comparison of thermal death rate kinetic properties was achieved by determining the logarithmic D values from a best fit straight line constructed between the data points (log_{10} cell concentration against holding time). These estimated D values were illustrated in Table 4.20 .

Table 4.20 Approximate D values for S and R-colony forms of *L. monocytogenes*, calculated by plotting a best fit straight line through the curved data points of Log_{10} number of cell survivors against holding time at either 56°C or 60°C.

Colony form *L. monocytogenes*	**D and 1/k value at 56°C[c]**		**D and 1/k value at 60°C[d]**	
	37°C (110 rpm)[a]	**41°C (0 rpm)[b]**	**37°C (110 rpm)**	**41°C (0 rpm)**
S-form (NCTC 11994)	6.04‡	22.87	0.603	3.51
R-form (NCTC 9863)	6.0	19.29	0.678	3.57
R-form (NRB5)	7.2	24.48	1.108	4.45

‡ Estimated D value in minutes at 56°C holding
[a,b] Cultivation conditions prior to heat treatent at 56°C
[c,d] Heat treatment temperature

The present and previous thermal study has illustrated that rough colony types (i.e. NRB2 and NRB5) exhibit a different thermal death rate curve to the standard smooth form. Furthermore, these R-surface colony types appear more heat tolerant compared to the S-form surface cultures (NCTC 11994 and 9863), which is primarily due to the presence of a tail section in the latter part of the survivor curve.

In summary, the series of thermal inactivation experiments have been planned and implemented in such a manner as to establish whether or not a variation in the heat

resistance of *L. monocytogenes* can occur by simply modifying the cultivation, heat inactivation, dilution, enrichment and enumeration conditions. It has been demonstrated that when certain environmental or cultural parameters are optimised, a substantial increase in the thermotolerance of *L. monocytogenes* can be achieved.

The parameters which appear to influence subsequent heat resistance ability include; the strain type (serotype 4b strains being more heat resistant than serotype 1a); colony morphological form (rough form being more heat tolerant); cultivation conditions prior to heat treatment (cells grown at 41°C in a stationary position being more heat resistant than those cultivated at 37°C under orbital conditions) and the heat inactivation temperature and exposure time (obviously the greater the holding temperature and the longer the exposure the greater the cell destruction).

While conditions shown to significantly improve the recovery of sublethally injured cells include, the direct plating or enrichment of heat treated cells (a greater recovery of thermally treated cells was achieved when the cell were enriched); the duration of the enrichment period (more recovery of damaged cells was achieved after 3 days liquid resuscitation compared to just 16 hours enrichment at 30°C); the composition of the enumeration plating medium (a greater recovery of heat subjected cells was achieved on non-selective plating media, however the effect of media composition appeared more significant for cells which had been directly plated without prior enrichment) and the duration of incubation of the plating medium prior to enumeration (3 days storage of seeded plates provided enhanced recovery of heat subjected *L. monocytogenes* cells.

It has been established that a variation in heat resistance exists among individual strains of *L. monocytogenes*, with the serotype 4 b strains (NCTC 11994 and NCTC 9863) being consistently recovered in greater numbers at all heat treatment temperatures compared to the test serotype 1a strain (NCTC 10357). Furthermore the plot of $\log_{10}$ cell number of survivors against holding time does not conform to a logarithmic death rate, instead a thermal death rate survivor curve is produced. The normal smooth colony forms exhibit a shoulder portion preceding an accelerating death rate on heating at a lethal temperature, while the R-forms differ slightly by having a tail section at the end of their survivor curves. Thermal death rate curves can

be linearised by application of the formula $(\log No - \log N)^a = kt + c$. However, linearisation will only occur for survivor curves which have a shoulder section only.

Once linearised, an indication of the time required for a 1 log reduction in cell number can be achieved by calculating the reciprocal of the thermal death rate constant k. This 1/k value (derived at a fixed heating temperature) is an analagous parameter to that of the logarithmic decimal reduction time value (D value). Determination of the 1/k values will subsequently permit the comparison of thermotolerance capabilities between strains which had been cultivated and heat treated at fixed bactericidal temperatures under similar conditions. Serotype 4b strains of *L. monocytogenes* (NCTC 11994 and 9863) were consistently more heat resistant than the 1a strain, irrespective of the growth, heat treatment and enumeration conditions employed.

Over the course of these thermal experiments it was observed that the colony form of *L. monocytogenes* could vary compared to that of the reference smooth colony type (i.e. normal NCTC cultures). These pleomorphic colony forms were designated as rough (R-forms) and could be distinguished from the parent S-form by differences in colony shape (outer margin, elevation, shape etc.), colour, opacity etc. which emerged on the agar surface. These pleomorphic surface cultures were composed of either a homogenous distribution of long cell chains ($\approx$ 60 μm in length) or a mixture of single, paired cells and/or long chains. Once identified and isolated to purity, these R-forms were shown to vary in stability. Earlier work revealed that the long chain cell arrangements present in the R-colony form was not simply an agar surface phenomenon but also existed in broth cultures.

These pleomorphic surface cultures emerged under conditions of heat stress, where the *Listeria* cells were cultivated at 41°C prior to heat treatment. Rough colonies did occasionally emerge when the cells were initially cultivated at 37°C prior to heating, but the frequency of conversion was far inferior to that of cell growth at the elevated temperature of 41°C. All three test *L. monocytogenes* strains (NCTC 11994, 9863 and 10357) supported the development of rough colony types. Non heat treated test cultures (irrespective of the cultivation conditions) did not vary in colony morphology throughout the entire series of thermal studies.

4.1.3 Identification of a suitable resuscitation broth and recovery conditions for the detection of heat subjected *Listeria* cells from contaminated infant milk formulae.

The information supplied from the previous series of thermal studies were used to construct a standard recovery protocol, which would improve the chances of recovering and/or detecting *L. monocytogenes* which had been subjected to a heat treatment. The objective of the following series of experiments was to identify an improved enrichment method for the recovery of healthy and sublethally injured *Listeria* cells and to establish whether or not a greater recovery of heat subjected cells would occur by implementing conditions previously shown to enhance thermotolerance. Indeed, the identification of a suitable enrichment medium coupled with the knowledge gained from the previous thermal experiments (i.e. the optimum combination of enrichment and enumeration conditions post heat treatment) should increase the chances of recovering thermally treated *L. monocytogenes* cells which may be present in dried infant milk formulae (IMF).

4.3.1 Determination of a fixed concentration of heat treated *Listeria* cells in a suitable suspension medium for subsequent enrichment broth evaluation.

The study was initiated by establishing culture and heat inactivation conditions which would provide a known concentration of heat subjected cells, in which the subculture medium had been removed and replaced by a neutral phosphate buffer. Moreover, in order to obtain a true reflection of the efficacy of each enrichment broth to recover thermally treated *Listeria* cells, it was deemed necessary to remove all traces of the subculture medium (e.g. TSYEB) from the cells prior to inoculation into each test resuscitation broth.

While the standard practice was be to simply resuspend the 24 hour TSYEB culture in an equivalent volume of 0.01 M PBS buffer solution and use this (or a dilution there

of) as the heating menstruum, the problem lay in the fact that the heat resistance of *Listeria* cells resuspended in 0.01 M PBS (acting as a heating menstruum) may be significantly lower than the same concentration of cells suspended in TSYEB. Inoculating a sample of heat subjected *Listeria* cells (e.g. holding at 56°C for 15 to 20 mins which was previously shown to reduce a *Listeria* cell population by 1 log fold in TSYEB) in 0.01 M PBS, may not guarantee the desired 1 $\log_{10}$ reduction in cell number as a greater number of cells may be destroyed. Therefore the objective of this particular part of study was to examine the variation in heat resistance response for S and/or R-form *Listeria* cells which were: suspended and heat treated at 56°C for 20 mins in 0.01 M PBS buffer solution prior to enumeration (and/or broth inoculation); suspended and heat treated at 56°C for 20 mins in TSYEB followed by resuspension in 0.01 M PBS buffer solution prior to enumeration (and/or broth inoculation); and suspended and heat treated at 56°C for 20 mins in TSYEB prior to enumeration (control).

The study was initiated by obtaining single colonies of S-from (NCTC 11994) and R-form (NRB2) on TSYEA and LSA plates by cultivation of seeded plates at 37°C for 24 hours. Separate duplicate 250 ml Erlenmeyer flasks containing 100 ml of TSYEB were inoculated with an isolated colony and then incubated at 37°C under orbital conditions (110 rpm) for 24 hours. The cell concentration of each 24 hour test culture was confirmed to be approximately 1.0×10^9 CFU ml^{-1} by measuring the O.D. at 440 nm and carrying out a total aerobic mesophilic count on TSYEA and LSA plates.

A decimal dilution series of this subculture was made in 0.01 M PBS and a 1 ml (using the 10^{-5} dilution) of this adjusted $_{440\ nm}$ subculture was aseptically inoculated into 100 ml TSYEB. This test culture was then cultivated at 37°C for 24 hours at 110 rpm, at which point the cell concentration (CFU ml^{-1}) was confirmed to be approximately 1.0×10^9 CFU ml^{-1} as described earlier. A waterbath was equilibrated at 56°C and the warming up period was calculated for each type of sterile heating menstruua (i.e. TSYEB and PBS) as described in previous thermal experiments (section 4.1.2.2.7).

The S and R form test cultures were heat treated in 2 ways. Firstly, the adjusted TSYEB test culture was pelleted , washed (x3) and resuspended in 0.01 M PBS. One ml aliquots of this resuspended test culture was aseptically transferred into a series of pre-labelled 28 ml McCartney bottles (H_0, T_0, T_5, T_{10}, T_{15}, T_{20}, T_{25}, T_{30}, T_{40}, T_{50}, T_{60} mins, C_1 and C_2) containing 9 ml of 0.01 M PBS. Secondly, 1ml of the adjusted test culture was transferred into the a duplicate set prelabelled 28 ml McCartney bottles containing 9 ml TSYEB (as labelled above).

Both sets of heating menstrua were submerged in the waterbath and held at 56°C for the designated sample times mentioned earlier. The warming up period for each heating menstruum was compensated for when determining holding times at 56°C. All heat treated samples were placed on ice prior to enumeration. The PBS heating menstruum sample bottles (containing suspended heat subjected *L. monocytogenes* cells) were enumerated on TSYEA and LSA via the spiral plating technique. The thermally subjected cells which had been heat treated in TSYEB were re-suspended in 0.01 M PBS post heat treatment and enumerated as described above. As a control, the heat treated TSYEB test cultures were plated out without subsequent resuspension.

All seeded plates were incubated for 3 days at 30°C post heat treatment. Identification of the emerging colonies was achieved by applying the standard set of identification tests (section 4.1.2.1.1). All seeded plates containing both uninjured and heat stressed cells were enumerated after 20 hours and 3 days incubation. Thermal death rate plots of $\log_{10}$ cell number of survivors against holding times were constructed for each heating menstruum.

Examination of the thermal death rate plots ($\log_{10}$ cell number of survivors against holding time values) showed that the heat treatment of *L. monocytogenes* cells suspended in 0.01 M phosphate buffered saline (PBS) significantly reduced (P 0.05) the heat resistance of the *Listeria* cells compared to cells which had been suspended and heat treated in the TSYEB control (Figures 4.42 and 4.43).

Furthermore, heat treatment of *Listeria* cells which were suspended in TSYEB with subsequent re-suspension in PBS (post heat treatment) resulted in a reduced recovery of heat treated *L. monocytogenes* at each holding time interval compared to the plate

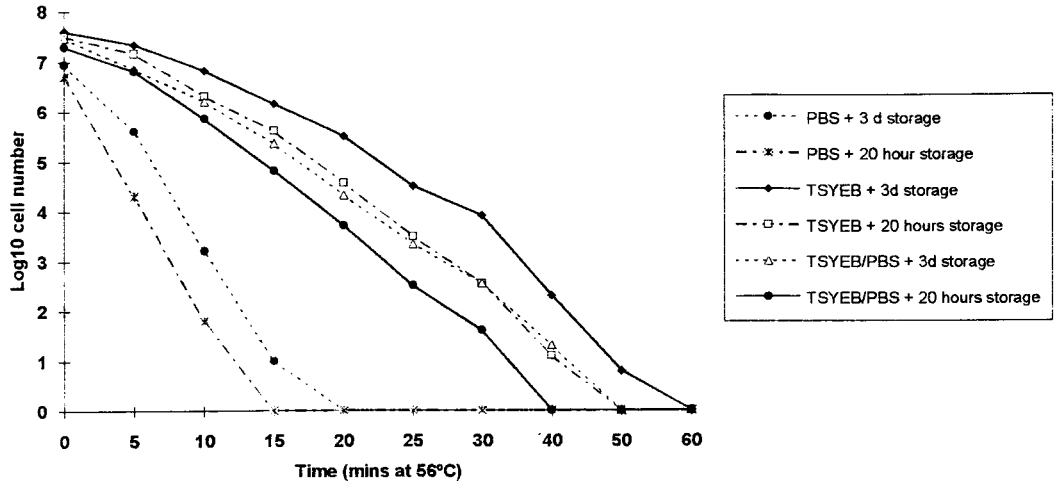


Figure 4.42 Thermal death rates for the smooth colony form (NCTC 11994) of *L. monocytogenes* which had been heat treated at 56°C in 0.01 M PBS, TSYEB and TSYEB with subsequent transfers to PBS. The heat treated test cultures were plated onto TSYEA and enumerated after 20 hours and 3 days.

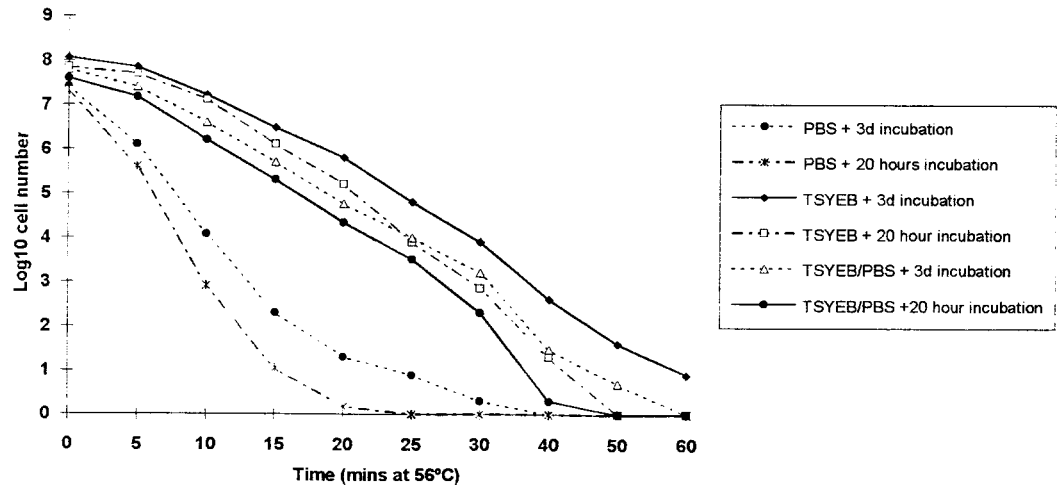


Figure 4.43 Thermal death rates for the rough colony form (NRB2) of *L. monocytogenes* which had been heat treated at 56°C in 0.01 M PBS, TSYEB and TSYEB with subsequent transfers to PBS. The heat treated test cultures were plated onto TSYEA and enumerated after 20 hours and 3 days.

counts of heat treated test cultures which had been simply heated in TSYEB without any additional transfers. Moreover, cells suspended in TSYEB without subsequent transfers were more thermotolerant than if the same concentration of cells were suspended in PBS on heating or heat treated in TSYEB followed by resuspension in PBS prior to enumeration. This observation was true for both the S and R colony forms under study. Thermal death rate curves were obtained for both surface culture forms. The R-colony form differed from the smooth in that it exhibited a shoulder and a tail section.

While this heating study has demonstrated that the heat inactivation of PBS suspended *Listeria* cells was more pronounced than in TSYEB, it also illustrates that an additional 48 hours incubation of seeded plates significantly increased the recovery of heat treated cells (especially at the longer exposure times). It appeared that the subsequent spinning and washing of heat subjected cells in PBS post heat treatment had a greater bactericidal effect on the suspended *Listeria* cells compared to the plating out of TSYEB heat treated test cultures.

Table 4.21 illustrates the *estimated* decimal reduction time values (mins) for the S-form (NCTC 11994) and R-form (NRB2) *L. monocytogenes* which had been suspended and heated at 56°C in either 0.01 M PBS or TSYEB (and/or with subsequent transfers to PBS from TSYEB).

Colony form	0.01 M PBS[a]		TSYEB[b]		TSYEB/0.01 M PBS[c]	
	TSYEA (20h)[‡]	TSYEA (3d)[†]	TSYEA (20h)[‡]	TSYEA (3d)[†]	TSYEA (20h)[‡]	TSYEA (3d)[†]
S-form (NCTC 11994)	2.04	2.52	6.04	7.53	5.26	6.03
R-form (NRB2)	2.8	4.24	6.32	7.96	5.47	6.9

[a] cells suspended in PBS and heated at 56°C prior to plating onto TSYEA.
[b] cells suspended in TSYEB and heated at 56°C prior to plating onto TSYEA.
[c] cells suspended in TSYEB and heated at 56°C , followed by resuspension in PBS prior to plating onto TSYEA.
[‡] plating media incubated for 20 hours prior to enumeration
[†] plating media incubated for 3 days prior to enumberation

As it was not possible to linearise the thermal death rate curve for the rough colony type (due to the shoulder and tail effect), an estimation of the decimal reduction time (mins) was achieved by plotting a best fit straight line through the data points (log_{10} cell number of survivors against holding time). The results of these D value calculations are illustrated in Table 4.21. The tabulated results suggests that it took 2.52, 7.53 and 6.03 mins at 56°C to reduce the S-form cell population by one log when these cells were heated in 0.01 M PBS, TSYEB and TSYEB with subsequent transfers to 0.01 M PBS respectively (after 3 days storage of plates). The information illustrated in this table further suggests that the R-form (NRB2) was more heat tolerant as it took 4.24, 7.96 and 6.9 mins at 56°C to obtain the same decimal reduction in cell number.

On the basis of the information supplied in Table 4.21, it was decided to heat treat the *Listeria* cells in TSYEB followed by subsequent washing and resuspension in 0.01 M PBS. Therefore, in order to obtain an initial inoculum of 10^1 to 10^2 heat treated cells ml^{-1} in each enrichment medium, it would be necessary to hold the rough and smooth forms in TSYEB at 56°C for 6.03 mins and 6.9 mins respectively (resulting in a 1 log reduction in cell number), making a serial dilution of the heating menstruum and aseptically transferring 1 ml of the 10^{-3} dilution into each enrichment medium (which contained 100 ml of medium).

Thus, by holding a fixed cell concentration (CFU ml^{-1}) of NCTC 11994 and NRB2 in TSYEB at 56°C for 6.03 and 6.9 mins respectively (and resuspending the cells in PBS) it is possible to obtain a known concentration of heat treated cells in each enrichment medium.

4.1.3.2 Identification of the optimum enrichment medium for the recovery of heat subjected *Listeria* cells.

The objective was to simply inoculate a fixed concentration of heat subjected cells (as defined in the previous study) into a series of non-selective and selective enrichment broths and assess the efficiency of each broth in resuscitating these treated cells by way of total aerobic mesophilic counts taken at regular intervals over a 24 hour

observation period. Heat subjected samples were spirally plated onto LSA (oxford formulation) and TSYEA plates where they were incubated for 3 days at 30°C prior to enumeration.

The study was initiated by streaking *L. monocytogenes* NCTC 11994 and NRB2 to single colonies on TSYEA and LSA plates, the plates being incubated at 37°C for 24 hours. The identity of 3 randomly isolated colonies was confirmed as described previously. A TSYEB test culture of 10^9 CFU ml^{-1} was achieved for both culture forms as described in section 4.1.2.2.13. A 1 ml aliquot of TSYEB test culture was transferred into duplicate 28 ml McCartney bottles containing 9 ml TSYEB. A warming up period was allowed for prior to equilibration of the heating menstruua at 56°C. The smooth and rough heat treated test cultures were removed after 6.03 and 6.9 mins respectively and stored on ice. The cells were centrifuged, washed (x3) and resuspended in 0.01 M PBS. Making a 10^{-3} dilution of this PBS resuspension, 1 ml was inoculated into the following series of enrichment broths:

- tryptone soya broth (TSB)
- tryptone soya broth supplemented with 0.25% glucose (TSGB)
- tryptone soya broth supplemented with 0.6% yeast extract (TSYEB)
- tryptone soya broth supplemented with 0.25% glucose and 0.6% yeast extract (TSYEGB)
- brain heart infusion broth (BHIB)
- brain heart infusion broth supplemented with 0.25% glucose (BHIGB)
- brain heart infusion broth supplemented with 0.25% glucose and 0.6% yeast extract (BHIYEGB)
- nutrient broth no.1 (NB)
- nutrient broth no.1 supplemented with 0.25% glucose (NGB)
- *Listeria* enrichment broth base (FDA approved formulation- Oxoid CM862) (LEB-FDA)
- *Listeria* enrichment broth with antibiotic supplements (FDA approved -Oxoid CM862&SR141) (LEBs-FDA)
- *Listeria* enrichment broth base (USDA-FSIS approved UVM1 formulation) (LEB-USDA)
- *Listeria* enrichment broth with antibiotic supplement (USDA-FSIS approved UVM1 formulation) (LEBs-USDA)
- tyndallised SMA Gold Cap (infant milk formula) (SMA Gold)

The various enrichment media were incubated under orbital conditions (50 rpm) at 30°C. Although aerobic resuscitation of heat treated cells was shown to be an inferior recovery technique compared to that of stationary enrichment, it was decided to

incubate the flasks under rotary conditions in order to obtain a homogeneous distribution of cells in each enrichment flask. Sterile enrichment media controls were run in parallel with the test enrichment cultured media. A total aerobic mesophilic count (CFU ml^{-1}) was carried out at regular intervals (0, 5, 12 and 24 hours) by spirally and spread plating the enriched samples onto TSYEA and LSA plates.

Table 4.22 Recovery of heat treated *L. monocytogenes* cells (Smooth form-NCTC 11994) from a variety of enrichment broths over a 24 hour sample period where the enrichment media were agitated under orbital conditions (50 rpm) at 30°C.

Enrichment Broths‡	Total Aerobic Mesophilic Count (CFU ml^{-1})							
	0 hr†		5 hr		12 hr		24 hr	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
TSB	$9.6x10^2$	$2.2x10^2$	$1.06x10^3$	$8.9x10^2$	$1.85x10^6$	$1.92x10^6$	$1.07x10^9$	$1.09x10^9$
TSGB	$7.9x10^2$	$1.8x10^2$	$1.08x10^3$	$9.16x10^2$	$2.02x10^6$	$2.08x10^6$	$1.7x10^9$	$1.47x10^9$
TSYEB	$9.0x10^2$	$3.2x10^2$	$1.17x10^3$	$9.7x10^2$	$2.27x10^6$	$1.82x10^6$	$1.78x10^9$	$1.76x10^9$
TSYEGB	9.2x102	$1.8x10^2$	$9.9x10^2$	$9.7x10^2$	$2.0x10^6$	$2.04x10^6$	$1.54x10^9$	$1.6x10^9$
BHIB	$7.4x10^2$	$1.5x10^2$	$1.0x10^3$	$6.1x10^2$	$1.72x10^6$	$1.8x10^6$	$1.51x10^9$	$1.34x10^9$
BHIGB	$8.7x10^2$	$2.2x10^2$	$1.01x10^3$	$6.9x10^2$	$1.97x10^6$	$1.53x10^6$	$4.98x10^8$	$4.33x10^4$
BHIYEGB	$9.1x10^2$	$2.9x10^2$	$1.12x10^3$	$8.6x10^2$	$2.83x10^6$	$2.63x10^6$	$1.37x10^9$	$1.34x10^9$
NB	$8.5x10^2$	$2.6x10^2$	$1.22x10^3$	$1.1x10^3$	$2.43x10^6$	$2.23x10^6$	$4.49x10^8$	$1.55x10^9$
NGB	$9.5x10^2$	$2.0x10^2$	$1.15x10^3$	$1.05x10^3$	$2.05x10^6$	$1.98x10^6$	$2.32x10^8$	$4.17x10^8$
LEB (FDA)	$8.0x10^2$	$1.7x10^2$	$7.69x10^2$	$6.1x10^2$	$6.5x10^5$	$6.3x10^5$	$4.1x10^8$	$2.11x10^8$
LEBs (FDA)	$7.4x10^2$	$1.4x10^2$	$5.7x10^2$	$5.27x10^2$	$3.26x10^4$	$2.68x10^4$	$4.48x10^8$	$3.0x10^8$
LEB (USDA)	$7.5x10^2$	$1.1x10^2$	$1.1x10^2$	$1.1x10^2$	$1.61x10^4$	$1.49x10^4$	$2.89x10^8$	$2.8x10^8$
LEBs (USDA)	$6.2x10^2$	$5.0x10^1$	$5.5x10^1$	$2.7x10^1$	$6.0x10^3$	$5.3x10^3$	$3.1x10^8$	$3.13x10^8$
SMA Gold	$9.0x10^2$	$2.7x10^2$	$1.05x10^3$	$9.1x10^2$	$2.79x10^6$	$2.71x10^6$	$1.58x10^9$	$1.63x10^9$

† Sampling time period for each enrichment broths (broths were orbitally agitated at 50 rpm)
‡ Refer to above text for a description of the abbreviated enrichment broths.

From Table 4.22 it can be seen that the most suitable enrichment media were defined media without antibiotic or dye supplements. Surprisingly, the lowest recovery of treated cells occurred in the two recommended enrichment broths (FDA and/or USDA-FSIS formulations), irrespective of whether or not they had been supplemented with antibiotics (P0.05). However, the addition of the recommended antibiotic supplements to both primary enrichment broths did further reduce the recoverability of heat subjected *Listeria* cells (i.e. efficiency of detection decreased). Over the first 12 hours of resuscitation, fewer heat treated *Listeria* cells were recovered on the selective plating medium LSA compared to the total counts (CFU

ml^{-1}) enumerated on TSYEA plates. The supplementation of BHI and TS broths with either glucose and/or yeast extracts did not significantly increase the recovery of thermally injured cells. However, the supplementation of enrichment broths with yeast extract did appear to provide conditions suitable for the recovery of consistently high number of previously heat treated cells (e.g. TSYEB and BHIYEGB).

Infant milk formula (i.e. SMA Gold Cap) appeared to be an efficient enrichment medium in the subsequent recovery of healthy and heat injured *Listeria* cells. From the information supplied in Table 4.22, the use of a non-selective primary enrichment stage prior to exposing the heat subjected cells to a cocktail of selective antibiotics and/or dyes would be recommended. A 10 hour pre-selective enrichment in either TSYEB or BHIYEGB could potentially improve the efficiency of subsequent selective stages in detecting and/or resuscitating heat treated *L. monocytogenes* cells.

A similar trend in the recovery of heat treated R-form (NRB2) cells from the various enrichment media emerged, where significantly fewer cells were resuscitated in enrichment broths which had been supplemented with antibiotics and dyes (Table 4.23).

An overall reduced microbial count for NRB2 was achieved in the enrichment broths at the 0 hours sample period compared to that of the S-form (which was probably due to the 24 hour cell concentration of the R-form test culture not being standardised). The 24 hour cell concentrations for the smooth and rough form of *L. monocytogenes* were shown to be 2.35×10^9 and 7.8×10^8 CFU ml^{-1} respectively prior to heat treatment. However, this difference in initial cell concentration (between both cultural forms) did not influence the heat resistance response exhibited by the individual colony types (i.e. a standardised cell concentration was achieved in the separate sets of enrichment broths for each colony form).

Table 4.23 Recovery of heat treated *L. monocytogenes* cells (rough form-NRB2) from a variety of enrichment broths over a 24 hour sample period where the enrichment media were resuscitated under orbital conditions (50 rpm) at 30°C.

Enrichment Broths‡	Total Aerobic Mesophilic Count (CFU ml^{-1})							
	0 hr†		5 hr		12 hr		24 hr	
	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA	TSYEA	LSA
TSYEB	$5.4x10^{-}$	$1.91x10^{-}$	$7.9x10^{-}$	$6.9x10^{-}$	$1.65x10^{0}$	$1.53x10^{0}$	$1.71x10^{9}$	$1.77x10^{9}$
BHIYEGB	$5.9x10^{2}$	$1.8x10^{2}$	$7.5x10^{2}$	$5.8x10^{2}$	$1.55x10^{6}$	$1.49x10^{6}$	$1.66x10^{9}$	$1.55x10^{9}$
LEB (FDA)	$4.3x10^{2}$	$1.01x10^{2}$	$5.6x10^{2}$	$4.8x10^{2}$	$6.4x10^{5}$	$6.1x10^{5}$	$3.9x10^{8}$	$3.81x10^{8}$
LEBs (FDA)	$4.1x10^{2}$	$7.5x10^{1}$	$3.9x10^{2}$	$3.4x10^{2}$	$2.86x10^{4}$	$2.5x10^{4}$	$3.62x10^{8}$	$3.54x10^{8}$
LEB (USDA)	$3.7x10^{2}$	$8.6x10^{1}$	$1.65x10^{2}$	$1.54x10^{2}$	$1.91x10^{4}$	$1.72x10^{4}$	$2.16x10^{8}$	$2.25x10^{8}$
LEBs (USDA)	$3.4x10^{2}$	$3.0x10^{1}$	$2.6x10^{1}$	$1.5x10^{1}$	$5.6x10^{3}$	$5.2x10^{3}$	$1.49x10^{8}$	$1.41x10^{8}$
SMA Gold	$6.1x10^{2}$	$1.75x10^{2}$	$7.5x10^{2}$	$6.1x10^{2}$	$1.57x10^{6}$	$1.50x10^{6}$	$1.59x10^{9}$	$1.61x10^{9}$

† Sampling time period for each enrichment broths (broths were orbitally agitated at 50 rpm)

‡ Refer to text for full description of these abreviated enrichment broths.

4.1.3.3 The use of a non-selective primary enrichment stage to improve the efficacy of the standard USDA-FSIS and/or FDA *Listeria* selective enrichment techniques.

The objective of this study was to establish whether or not the use of a non-selective primary enrichment broth (TSYEB) in conjunction with the standard selective enrichment FDA and USDA-FSIS broths would increase the recovery of heat treated cells of *L. monocytogenes* exposed to a sub-lethal temperature.

It was previously demonstrated (enrichment study one) that a non selective enrichment broth (e.g. TSYEB or BHIYEGB) provided greater recovery of heat subjected *Listeria* cells compared to resuscitation broths which were supplemented with antibiotics and dyes. Therefore the efficacy was examined of TSYEB (used as a primary enrichment stage prior to resuscitation in the recommended *Listeria* enrichment broths) in recovering *Listeria* cells from heat treated test cultures held for 3 different time periods at 56°C. Thus, each heat treated test culture should have a different concentration of survivors. The 3 heat treated samples would then be

inoculated into TSYEB, LEB (FDA) and LEB (USDA) and then enriched for 10 hours prior to inoculation into the following broths:

- a sample from the 10 hour enriched TSYEB was transferred to LEB (FDA and/or USDA) primary enrichment broths.
- a sample from the 10 hour enriched LEB (FDA) was transferred into LEB secondary enrichment broth (again FDA-Modified Frazer broth formulation).
- a sample from the 10 hour enriched LEB (USDA-UVM1 formulation) was transferred into LEB secondary enrichment broth (USDA- UVM11 formulation).

The cell concentration in each 10 hour primary enrichment broth was determined via total aerobic mesophilic counts (which allowed a comparison of cell recovery potential to be made). The ability of each secondary enrichment stage to resuscitate heat treated cells was monitored by way of a total aerobic mesophilic count at the 0 hour and 13 hour enrichment period.

The investigation was initiated by inoculating duplicate TSYEB test media with a single colony of the S-form (NCTC 11994) or the R-form (NRB2). These subculture media were cultivated at 37°C for 24 hours under orbital conditions (110rpm).

The concentration of the 24 hour subculture, confirmed to be approximately $1.0x10^9$ CFU ml^{-1} (as described previously), was pelleted, washed (x3) and resuspended in 0.01 M PBS . A 1 ml aliquot of the 10^{-5} dilution (using 0.01 M PBS as diluent) was transferred into duplicate 250 ml Erlenmeyer flasks containing 100 ml TSYEB . The S and R form TSYEB test cultures were cultivated at 37°C for 24 hours at 110 rpm (a total aerobic mesophilic count was carried out at this point). A 1 ml aliquot of the respective test cultures was transferred into a set of three pre-labelled 28 ml McCartney bottles (T_{15}, T_{20} and T_{35} mins, C1 and C2, where C1 and C2 is the non heat treated test culture and sterile heating menstruum controls respectively) containing 9 ml TSYEB.

A standard heating curve (°C versus time values) was constructed and the warming up time period to 56°C was determined by interpolation. When the submerged heat inactivation bottles reached equilibrium at 56°C, the sample bottles were removed from the waterbath at the designated holding time intervals mentioned above. The heating menstruum was centrifuged, washed and resuspended (x3) in 0.01M PBS.

A 0.2 ml aliquot of each heat treated time sample (i.e. T_{15}, T_{20} and T_{35} mins) was aseptically inoculated into; 20 ml TSYEB; 20 ml LEB with antibiotic supplement added (FDA formulation); and 20 ml LEB with antibiotic supplement added (USDA formulation i.e. UVM1).

These primary enrichment broths were incubated for 10 hours at 35°C in a stationary position and a total aerobic mesophilic count (CFU ml^{-1}) was carried out at this 10 hour enrichment time period.

A 0.1 ml aliquot from the 10 hour primary enrichment broths was aseptically inoculated into the following secondary enrichment broths:

- 0.1 ml aliquots of each TSYEB primary enrichment broth (T_{15}, T_{20} and T_{35} mins samples at 56°C) was inoculated into 20 ml of sterilised LEB (FDA) and LEB (USDA) primary enrichment broths.
- 0.1 ml aliquots of each LEB (FDA) primary enrichment broth (T_{15}, T_{20} and T_{35} mins samples at 56°C) was inoculated into 20 ml of sterilised LEB (FDA= Modified Frazer broth) secondary enrichment broths.
- 0.1 ml aliquots of each LEB (USDA) primary enrichment broth (T_{15}, T_{20} and T_{35} mins samples at 56°C) was inoculated into 20 ml of sterilised LEB (USDA=UVM11 formulation) secondary enrichment broths.

This series of primary/secondary enrichment broths was incubated at 35°C for 13 hours in a stationary position. A total aerobic mesophilic count (CFU ml^{-1}) was carried out on TSYEA and LSA (Oxford formulation) after 0 and 13 hours enrichment for all the enriched heat treated test cultures. Samples were diluted and enumerated via the spread, pour and spiral plating techniques. Sterile enrichment controls were employed and analysed in addition to the test enrichment media. All plating media were incubated for 3 days at 30°C prior to enumeration. Three colonies were selected from each test strain (NCTC 11994 and NRB2) for identification.

It took 2.37 mins for the TSYEB heating menstruum to reach the holding temperature of 56°C (i.e. from 24.1°C to 56°C). Table 4.24 confirmed that a significant reduction in *L. monocytogenes* cell concentration was achieved in the heating menstruum prior to inoculation of primary enrichment broths. A further 1:100 dilution of the heating menstruum occurred when 0.2 ml aliquots are transferred into

20 ml of primary enrichment broths and this low concentration of cells was used to test the efficiency of the 3 primary enrichment broths in recovering the heat subjected cells.

Table 4.24 illustrates the degree of cell destruction at each holding time period at the lethal temperature of 56°C. The *Listeria* cells were initially cultivated at 37°C (110 rpm) for 20 hours prior to heat treatment.

Holding time at 56°C (mins)	Total Aerobic Mesophilic Count(CFU ml^{-1}) on TSYEA plates	
	Smooth form (NCTC 11994)	Rough form (NRB2)
H_0†	$3.7x10^8$	$2.9x10^8$
T_{15}	$3.69x10^5$	$3.49x10^5$
T_{20}	$9.4x10^3$	$1.14x10^4$
T_{35}	$2.9x10^2$	$5.5x10^2$
† The cell concentration prior to heating		

Greater recovery of heat treated R and S form cells was achieved in the TSYEB (primary enrichment broth) at both the 0 and 10 hour sample time periods (Table 4.25). NRB2 was detected in greater numbers compared to the smooth colony form. There was no significant difference in the recovery of cells from either primary selective enrichment medium (P0.05). Within 10 hours, the non selective enrichment gave significantly increased (up to 10^2 fold) cell numbers compared to seletive enrichment broths.

Considering that the T35 mins sample produced the lowest numbers of uninjured and heat stressed cells on primary enrichment, the development of this heat treated sample was followed in the secondary enrichment stage (Table 4.24).

Table 4.25 Recovery of heat treated *Listeria* cells (expressed in terms of total aerobic mesophilic count-CFU ml^{-1}) in 3 primary enrichment broths after 0 and 10 hours enrichment.

Enrichment medium	Holding time at 56°C (mins)	0 hr Enrichment		10 hr Enrichment	
		NCTC 11994[a]	NRB2[b]	NCTC 11994	NRB2
TSYEB	15	$2.6x10^3$	$4.6x10^3$	$1.3x10^5$	$6.7x10^7$
	20	$9.3x10^1$	$1.05x10^2$	$3.6x10^2$	$4.6x10^5$
	35	NDx1	2.0x1	$8.6x10^2$	$7.1x10^3$
LEB (FDA)	15	$3.5x10^3$	$5.6x10^3$	$2.6x10^4$	$1.53x10^5$
	20	$1.2x10^2$	$2.1x10^2$	$2.53x10^3$	$6.1x10^3$
	35	NDx1	NDx1	$2.4x10^2$	$4.0x10^2$
LEB (USDA)	15	$3.15x10^3$	$4.36x10^3$	$1.9x10^4$	$8.46x10^5$
	20	$8.6x10^1$	$1.3x10^2$	$3.3x10^2$	$9.0x10^3$
	35	NDx1	NDx1	$2.0x10^1$	$1.2x10^3$

[a,b] Represents the total aerobic mesophilic count (CFU ml-1) for S-form and R-form respectively

Table 4.26 shows that primary enrichment in a non-selective medium improved recovery of heat treated *L. monocytogenes* cells. However, this brief study also demonstrated that the recommended selective enrichment broths would recover heat subjected cells that had been heated for 35 mins at 56°C. Having established that non-selective primary enrichment increased the recovery of injured cells in subsequent selective enrichment stages, the next experiment would involve heat treating both S and R colony forms at a pasteurisation temperature having initially cultivated the cells under conditions known to enhance *L. monocytogenes* heat resistance ability. Samples from these heat treated test cultures (e.g. from 0 to 30 mins at 62.8°C) would then be subjected to the standard enrichment techniques (and to modified versions of these methods) in order to establish whether or not the implementation of parameters previously shown to help heat resistance would increase the recovery of treated cells compared to simply following the exact enrichment guidelines laid down by the governing bodies.

Table 4.26 illustrates the total aerobic mesophilic counts on TSYEA plates (CFU ml^{-1}) for secondary enriched smooth form (NCTC 11994) which had been initially heat treated at 56°C for 35 mins and enriched in a variety of primary enrichment broths prior to inoculation into the secondary enrichment media.

Primary enrichment medium†	Secondary enrichment medium	Enrichment period (CFU ml^{-1})	
		0 hour at 35°C	13 hour at 35°C
TSYEB	LEB (FDA) One	$5.5x10^{3}$	$9.2x10^{6}$
TSYEB	LEB (USDA) One[a]	$4.0x10^{3}$	$7.6x10^{6}$
LEB (FDA) One	LEB (FDA) Two[b]	$8.3x10^{2}$	$2.01x10^{5}$
LEB (USDA) One	LEB (USDA) Two[c]	$7.7x10^{2}$	$1.28x10^{5}$

† illustrates the primary enrichment media which were stored for 10 hours prior to subsequent transfer to the secondary stage.
a Listeria enrichment broth, a UVM1 formulation and USDA-FSIS approved method
b Listeria enrichment broth two, a modified fraser broth formulation and FDA approved method
c Listeria enrichment broth two, a UVM11 formulation and USDA approved.

4.1.3.4 The efficacy of current methodologies in the detection of heat treated *Listeria* cells.

This investigation focused on evaluating the efficacy of current methodologies for the detection of heat subjected smooth (NCTC 9863) and rough (NRB2) colony forms of *L monocytogenes* which had been cultivated under various temperature and rotary conditions (prior to heat treatment). The cultural, heating, enriching and enumerating conditions previously shown to have influenced the recovery of greater numbers of heat treated *Listeria* cells were incorporated into the overall protocol.

The cells were subcultured in BHI broth prior to washing, adjusting and standardising ($A_{440\ nm}$) and inoculating into tyndallised infant milk formula (SMA Gold Cap). These test cultures were then cultivated in a stationary position for 24 hours at 41°C and 37°C. A measure of the final cell concentration in each test culture was achieved via a total aerobic mesophilic counts (CFU ml^{-1}). Indeed, the test cultivation conditions corresponded exactly to that of the previous subculture conditions, so where NRB2

was initially cultivated at 37°C in a stationary position then the same temperature and conditions were employed for the test cultivation of this R colony form.

The various test cultures were then subjected to different exposure times at the vat pasteurisation temperature of 62.8°C (i.e. H_0, H_3, T_0, T_3, T_5, T_{10}, T_{15}, T_{20}, T_{30}, and T_{35} mins, C_1 and C_2 where H, T, C_1 and C_2 represent the heating up period, the holding period, the non heat treated test culture and the sterile heating menstruum respectively). Obviously the longer the exposure time the greater the degree of cell injury, therefore samples heat treated at the longer holding times will contain low numbers of thermally treated *Listeria* cells which would test the resuscitation efficiency of subsequent enrichment and/or enumeration technique. The heating menstruum and diluent were of complimentary nutritional composition to that of the test cultivation medium (i.e. SMA Gold Cap).

Each heat treated test culture (exposed to the various holding times at 62.8°C) were subjected to various resuscitation and enumeration procedures. Firstly, duplicate samples were removed immediately post heat treatment and directly plated onto TSYEA and LSA (via spread, spiral and pour plating techniques) in order to quantify the degree of cell destruction obtained (CFU ml^{-1}) as a result of the individual heat exposures. Further duplicate samples were removed for subsequent enrichment purposes while the remainder of heat treated test culture were left to enrich in their respective heating menstrua (in a stationary position) for 24 hours at 30°C prior to future enrichment procedures.

The heat treated test culture samples removed from the heating menstruum (i.e. immediately after the heat exposure) were enriched in three ways:

a) duplicate 0.5 ml aliquots were aseptically transferred into 9.5 ml of non-selective primary enrichment broth (i.e. TSYEB) and then enriched for 10 hours at 30°C.
b) duplicate 0.5 ml aliquots were aseptically transferred into 9.5 ml of primary selective enrichment broth (FDA approved) where it was enriched for 24 hours at 30°C.
c) duplicate 0.5 ml aliquots were aseptically transferred into 9.5 ml of primary selective enrichment broth (USDA-FSIS approved) where it was enriched for 24 hours at 30°C.

After the 10 hour primary non-selective enrichment period and the 24 hour primary selective enrichment period (FDA and USDA methods) duplicate samples from each set of enrichment broths were loop inoculated onto both TSYEA and LSA plating media to confirm either the absence or presence of viable *L. monocytogenes*. In addition, 0.5 ml aliquots of the selective primary enrichment broths (FDA and USDA) were subculture into a series of secondary enrichment broths (Modified Frazers broth -FDA approved and UVM11-USDA/FSIS approved respectively). These separate secondary selective enrichment broths were incubated at 37°C for 48 hours and samples were removed after 24 and 48 hours for enumeration (i.e loop inoculation onto plating media to confirm absence or presence of viable *Listeria* cells).

To establish whether or not a greater recovery of sublethally injured and healthy *Listeria* cells was obtained by the addition of a non-selective primary enrichment stage, the 24 hour heating menstrua samples (which contained heat subjected cells and had been left to enrich post heat treatment) and the 10 hour TSYEB primary non-selective enriched samples were processed through the two standard selective enrichment techniques mentioned earlier (i.e. the FDA approved method and the USDA-FSIS approved method). It must be subjected that this entire cultivation, heat inactivation, dilution, enrichment and enumeration procedure was repeated for the smooth (NCTC 9863) and rough (NRB2) colony forms of *L. monocytogenes* which had been cultivated at 37°C and 41°C in a stationary position prior to heat treatment.

Thus, the objective of this enrichment experiment was to identify whether or not the standard recovery techniques could be made more efficient by the addition of either a non-selective enrichment stage (using either TSYEB or infant milk formula); whether these recommended recovery techniques could resuscitate both colony types of *L. monocytogenes* irrespective as to whether or not they were supplemented with a non selective primary enrichment stage; whether or not the variation in initial cultivation conditions and/or colony form influenced the recovery level of subsequently heat injured and healthy *L. monocytogenes* cells; and to confirm whether or not the vat pasteurisation process (holding at 62.8°C for 30 mins) was efficient at reducing a microbial population to a non detectable level having implemented conditions favourable to cell survival against sudden temperature extremes.

An initial cell concentration of ~$2.0x10^2$ CFU ml^{-1} was achieved in each of the 4 infant milk formula test cultures, i.e. $2.5x10^2$ CFU ml^{-1} (smooth at 35°C/ 0 hr), $2.37x10^2$ CFU ml^{-1}(smooth at 41°C/ 0 hr), $3.21x10^2$ CFU ml^{-1} (rough at 37°C/ 0 hr) and $2.15x10^2$ CFU ml^{-1} (rough at 41°C/ 0 hr), by inoculating 1 ml of the 10^{-4} dilution from the adjusted 24 hour subculture into the respective 100 ml of fresh test culture. These test cultures were then cultivated at the respective temperature for 24 hours prior to thermal inactivation at 62.8°C. As Figure 4.44 shows, it took 7.33 mins for the temperature of the heating menstruum to reach a holding temperature of 62.8°C and this warming up period was compensated for when determining the correct exposure times for the holding of each test culture at this lethal temperature.

The level of cell destruction (expressed as CFU ml^{-1}) resulting from heating of the different test cultures at 62.8°C (at the various exposure times) can be observed in Table 4.27. It was constructed from the number of cell survivors recovered (expressed as CFU ml^{-1}) by the direct plating technique at each holding time interval (mins at 62.8°C).

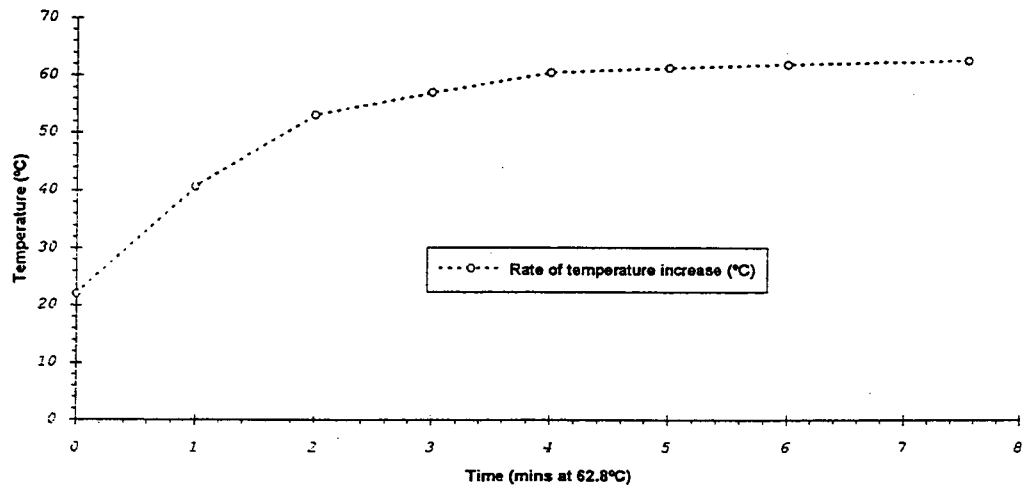


Figure 4.44 Rate of temperature increase in SMA Gold Cap from an initial room temperature of 22°C to a holding temperature of 62.8°C.

Moreover, Table 4.29 illustrates that the R-form appeared slightly more heat tolerant compared to the S-form, as a greater number of NRB2 survivors were present after 5 mins exposure at 62.8°C. Therefore, the direct plating technique did not recover any heat treated cells past an exposure time of 5 mins.

For convenience and to provide a clearer explanation of the results obtained from the various enrichment regimes the following abbreviations were made for the different test cultures of *L. monocytogenes*:

- $R_{41°C}$ and $R_{37°C}$ refer to NRB2 (rough colony form) cultivated in a stationary position at 41°C and 37°C respectively prior to heat inactivation at 62.8°C.
- $S_{41°C}$ and $S_{37°C}$ refer to NCTC 9863 (smooth colony form) cultivated in a stationary position at 41°C and 37°C respectively prior to heat inactivation at 62.8°C.

The information presented in Table 4.28 suggests that the two standard recovery techniques (i.e. the standard methods recommended by the Food and Drink Administration and the United States Department of Agriculture) are not as effective at recovering heat subjected cells of *L. monocytogenes* compared to the same recommended techniques having been supplemented with a non selective enrichment stage (either TSYEB or a reconstituted infant milk formula). The greatest recovery of heat treated cells (via the standard non-supplementative selective enrichment techniques) occurred when both surface culture types were cultivated at 41°C prior to heat treatment, however the maximum exposure tolerated by these cells was 5 mins at 62.8°C for the FDA and USDA approved selective enrichment methods.

Supplementation of these standard recovery techniques with either TSYEB or an infant milk formula pre non-selective enrichment stage, significantly improved the detection of heat treated cells at holding temperature of 62.8°C (P0.05). The best recovery of treated cells occurred when the test cultures were initially cultivated at the elevated temperature of 41°C as opposed to 37°C prior to heat treatment. Furthermore, the suspended *Listeria* cells were able to tolerate longer exposures at this lethal temperature when they were cultivated, heated and enriched in infant milk

formula, as seen by the emergence of R and S form colonies after 20 and 15 mins holding at 62.8°C.

Table 4.27 Recovery of heat treated smooth (NCTC 9863) and rough (NRB2) forms of *L monocytogenes* via the direct plating technique. The cells were separately cultivated at 41°C and 37°C in a stationary position prior to heat inactivation at 62.8°C and enumeration on TSYEA plates.

Heating conditions (mins)	Temp (°C) achieved	Total Aerobic Mesophilic Count (CFU ml^{-1}) on TSYEA.			
		41°C†		37°C†	
		Smooth form	Rough form	Smooth form	Rough form
H0	22	$6.1x10^7$	$7.5x10^7$	$5.2x10^7$	$7.3x10^7$
H3	57	$5.9x10^6$	$4.19x10^6$	$3.89x10^6$	$4.06x10^6$
T0	62.8	$8.8x10^4$	$5.3x10^5$	$6.6x10^4$	$3.26x10^5$
T3	62.8	$6.1x10^2$	$7.1x10^3$	$4.5x10^2$	$3.2x10^3$
T5	62.8	9.0x1	$4.9x10^2$	6.0x1	$2.05x10^2$
T10	62.8	NDx1‡	NDx1	NDx1	NDx1
T15	62.8	NDx1	NDx1	NDx1	NDx1
T20	62.8	NDx1	NDx1	NDx1	NDx1
T30	62.8	NDx1	NDx1	NDx1	NDx1
T35	62.8	NDx1	NDx1	NDx1	NDx1
T2a	22	NDx1	NDx1	NDx1	NDx1
C2b	22	NDx1	NDx1	NDx1	NDx1

† Represents the cultivation temperature of the test culture media prior to heat treatment.
‡ Represents failure to detect heat damaged cells in the undiluted heat treated test cultures (x1).
C2a,C2b Represent the total aerobic mesophilic counts obtained from the heating menstruua before and after heat treatment respectively.

The non selective enrichment of R and S surface colony types (prior to selective enrichment regimes) permitted the survival of heat subjected *Listeria* cells at 10 mins holding. The greater recovery of injured and healthy *Listeria* cells in SMA Gold Cap may have been due to the additional 14 hours resuscitation, the fact that the heating menstruum was left undisturbed post heat treatment, heat treated cells were more diluted 1:20 in the TSYEB and/or due to employing the same medium composition right through the experimental protocol (from cultivation to enrichment) putting less strain on the organism.

The level of detection of heat treated *Listeria* cells after the entire selective enrichment process was the same as the level of resuscitation obtained after the initial primary non selective enrichment (i.e no advancement or improvement in cell detection was made by adding the primary enriched cells to the recommended selective recovery techniques). The investigation did show that reconstituted infant milk formula was a suitable medium for the subsequent resuscitation of heat treated *Listeria* cells (of any morphological form).

There are several reasons why commercially available infant milk formula (IMF) should be analysed for the possible presence of this potential foodborne pathogen (section 2.1.1.5). Namely: *L. monocytogenes* is ubiquitously distributed in the environment, and it has been frequently isolated from foodstuffs such as dairy products; its normal route of infection is through food where it has been shown to exhibit a high fatality rate (70%) among susceptible groups (including infants); its pathogenicity and virulence characteristics; the under developed immune system of the consumer group (i.e. infants) and the susceptibility to low levels of infection; infant milk powders containing a low concentration of competing microflora (approximately $2.0x10^2$ CFU g^{-1} powder); the potential for this organism to increase its heat resistance whereby it may tolerate temperatures normally employed in the manufacture and drying of infant milk formula; the non-spore forming organism has been previously shown to survive the spray drying process; normal reconstitution of IMF does not eliminate the organism; the uncertainty of the infection dose required to initiate listeriosis (especially in infants); the potential for the organism to change its morphological appearance in IMF whereby it may have been previously misinterpreted as an inconsequential contaminant; and an in depth study of IMF for low levels of heat treated *Listeria* cells has not been previously carried out.

Furthermore, as the issue of heat subjected *Listeria* cells in food products has only recently been properly addressed and the search goes on for improved *Listeria* enrichment broths and recovery techniques capable of resuscitating these treated cells (section 2.1.1.7), this research, which employs improved enrichment techniques and modern identification systems to detect these potentially treated cells in contaminated IMF is deemed to be warranted.

Table 4.28 illustrates the end point detection (mins at 62.8°C) of heat stressed cells on TSYEA plates.

Enrichment medium employed at each resuscitation stage			End point determination of heat damaged *L. monocytogenes* cells (mins at 62.8°C)							
			24 h Primary Selective Enrichment				48 h Secondary Selective Enrichment			
Primary (non-selective)	**Primary** (selective) 30°C/24 hr	**Secondary** (selective) 37°C/48hr	$R_{41°C}$[†]	$R_{37°C}$	$S_{41°C}$	$S_{37°C}$	$R_{41°C}$	$R_{37°C}$	$S_{41°C}$	$S_{37°C}$
-	LEB (FDA)	LEB(FDA-MFB)	3	0	5	0	5	3	5	3
-	LEB (USDA-UVM1)	LEB (USDA-UVM11)	0	H3[‡]	H3	H3	5	3	5	3
TSYEB (10 hr)	LEB (FDA)	LEB(FDA-MFB)	10	5	10	5	10	5	10	5
TSYEB (10 hr)	LEB (USDA-UVM1)	LEB (USDA-UVM11)	10	5	10	5	10	5	10	5
SMA Gold (24 hr)	LEB (FDA)	LEB(FDA-MFB)	20	10	15	10	20	10	15	10
SMA Gold (24 hr)	LEB (USDA-UVM1)	LEB (USDA-UVM11)	20	10	15	10	20	10	15	10

[†] refers to the colony form (R for rough) and cultivation temperature prior to heat treatment (see text for a full description of the abbreviations)

[‡] H refers to heat injured cells recovered from the warming-up period only.

5. Microbiological analysis of reconstituted infant milk formula

Examination of the results presented in section 4 suggests that the heat resistance of *Listeria monocytogenes* may be significantly enhanced to a level where it may tolerate otherwise detrimental thermal treatment temperatures. This increased thermotolerance was shown to be related to the provision of certain growth, heat inactivation and enrichment conditions. Although the manufacture of infant milk formulae (IMF) employs high processing temperatures, it may be possible for low numbers of heat stressed *Listeria* cells to either survive the spray drying process or infiltrate the final milk powder by way of post-process contamination (section 2.3.2).

The aim of this series of studies was to determine the microbiological quality of 125 IMF, with particular attention being placed on the detection of thermally and/or osmotically stressed *Listeria* cells using enrichment media and conditions previously shown to encourage their resuscitation (section 4). While it has been documented that the normal indigenous bacterial population of IMF consists of mainly aerobic spore forming rods (section 2.3.2), this study examined the effect of infant formula reconstitution and subsequent storage temperatures (with or without additional pasteurisation), cooling method and biofilm build-up on inner walls of infant feeding bottles and teats, on the type, number and concentration of microorganisms present. Furthermore, whether or not the application of any particular combination of preparation and/or storage conditions would result in the recovery of non-spore forming *Listeria* cells.

As the identification *Bacillus* to species level has been shown to be extremely arduous, fraught with difficulty and often unacceptable (sections 2.21 and 2.2.2), initial studies focused on finding a reliable identification technique. Reconstituted infant milk formula and laboratory based media were also examined for the ability to support growth of enterotoxigenic *Bacillus* spp., which had been originally isolated from the IMF, at both refrigeration and ambient temperatures.

As demonstration of enterotoxin production by indigenous *Bacillus* spp. in reconstituted IMF has not been comprehensively investigated, this study employed two novel ultrafiltration systems which provided fat free extracts suitable for

enterotoxin analysis. A variety of carbohydrates (either naturally present or artificially added to IMF) were examined for the ability to support enterotoxin production by indigenous *Bacillus* spp. under various storage conditions.

The efficacy of normal cleaning and sterilisation methods (thermal and/or chemical) to reduce or eliminate microorganims in contaminated infant feeding bottles were assessed (where the feeding bottles contained either naturally or artificially contaminated IMF and were stored under conditions known to occur in the home and/or hospitals). The study was concluded with a case study, where a local hospital supplied reconstituted IMF samples for microbiological analysis.

5.1 Isolation and identification of *Bacillus* species from infant milk formulae.

The genus *Bacillus* is one of 13 bacterial genera which form endospores. These endospore-formers are separated into different genera by virtue of the fact that they differ morphologically, physiologically and genetically from one another (section 2.2.1). The assignment of individual species to an acceptable genus is often an extremely difficult and arduous task. Considering that there are currently 1,134 mesophilic *Bacillus* species alone, identification of a spore-forming organism to the genus level is only one in a number of stages on the route to species identification. Therefore, with the knowledge that there is often a fine boundary distinguishing bacterial genera and indeed species which contain endospores, a comprehensive study was undertaken to identify key physiological and morphological properties which would allow *Bacillus spp.*, isolated from infant milk formulae (IMF), to be confidently identified to species level.

The isolation of aerobic spore-formers from IMF was initiated by sterilising duplicate 500 ml Duran bottles containing 220 ml of water (to which was added 6 to 8 glass beads) and by autoclaving at 121°C for 15 mins. Care was taken to ensure that contamination of the infant milk powder did not occur by wearing sterilised vinyl gloves (soaked in 70% alcohol) and swabbing the outer package with 70% alcohol. The IMF was reconstituted by aseptically transferring 30 g of IMF into the 220 ml

pre-heated water, the contents of which were then shaken 25 times with an excursion of 30 cm in order to obtain a homogeneous suspension (the glass beads aided mixing).

The bottles were incubated at the desired temperature and time period. Enumeration and identification of the indigenous bacterial flora present at each time interval occurred by aseptically transferring 0.1 ml aliquots of the reconstituted IMF sample (and decimal dilutions there of) onto Tryptone Soya Agar -containing 0.6% Yeast Extract (TSYEA), Nutrient Agar No. 2 -containing 0.5 mg L^{-1} $MnSo_4xH_20$ (NAMS), Blood Agar No 2 - containing 7% defribrinated horse blood (BA), and *Bacillus cereus* Selective Agar (BCSA) containing the 50,000 IU polymyxin antibiotic supplement using the spread plate technique.

In addition to the spread plate technique (required to obtain isolated colonies), a Total Aerobic Mesophilic Count (CFU /g^{-1} IMF) was carried out by automatically diluting and dispensing a fixed reproducible volume (36 µl) of sample via the Spiral plater model B onto NA and BCSA in triplicate (section 3.5.6). All seeded plates were incubated in an inverted position for; 24 hours at 30°C for the detection of mesophilic species, for 24 and 48 hours at 55°C for thermophilic species; and/or for 6 to 8 days at 4-6°C for the detection of psychrophilic species. Infant formula samples which had been spirally plated were enumerated using the 10 cm counting grid (section 3.5.6).

Cultures obtained on the above plating media were examined for the following morphological and/or biochemical properties: TSAYE (Gram and catalase reactions, measure cell width and length (µm)), BCSA (egg-yolk reaction, and the development of a peacock blue discolouration), BA (haemolytic reaction and colony morphology (mm)) and NAMS (spore stain to determine spore shape and whether or not the endospore swells the sporangium).

While the growth characteristics exhibited by indigenous *Bacillus* on the above plating media aided subsequent identification, presumptive identification could only be achieved by establishing whether or not the unknown aerobic spore former belonged to the genus *Bacillus* and not to one of the other 12 bacterial genera capable of producing endospores. Differentiation between these endospore forming genera was

achieved by identifying key morphological, biochemical, physiological and genetically properties unique to each genera (Table 5.1).

Table 5.1 Key characteristics of the 13 bacterial genera known to produce endospores.

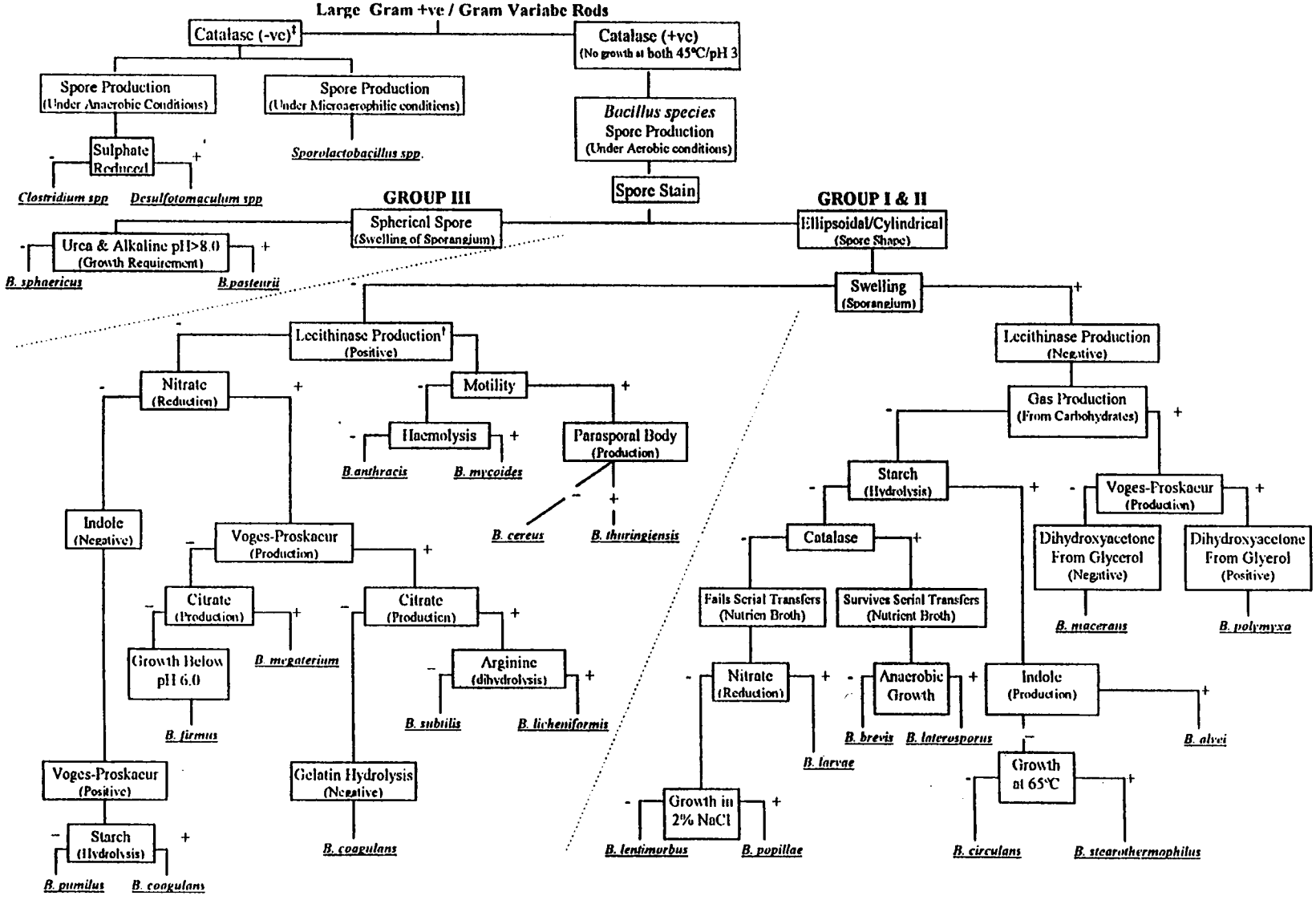
CLASSIFICATION WITHIN THE ENDOSPORE-FORMING GENERA		
GENUS	DESCRIPTION	KEY CHARACTERISTICS OF GENUS[1]
Alicolobacillus	Thermoacidophilic spp. which were previously known as *B. acidocaldarius*, *B. acidoterrestris* and *B. cycloheptanicus* Taxon is based on 16S rRNA catalogues where similarity of >98.9% was demonstrated among the 5 strains. Low similarity with Group 1 *Bacillus* spp.	Aerobic or facultative. Non-sporing under anaerobic conditions. Catalase +, Growth at 45°C and pH 3.0. Sulphur not used as energy source.
Amphibacillus	Forms spores under aerobic and anaerobic conditions. This distinguishes *Amphibacillus* from *Clostridium*, *Sporolactobacillus* and *Bacillus*.	Differs from *Alicobacillus* by not using ferrus iron, sulphide and sulphur as energy sources.
Bacillus	Species are arranged into 3 groups based on morphopogy and physiology. Apart from thermophilic and psychrotrophic spp. there are more than 1,100 mesophilic spp arranged into 19 spp.	Catalase usually produced, differs from *Amphibacillus* and *Alicobacillus* by not growing at both 45°C/pH 3
Clostridium	Species have %GC values of 22-55 (majority in range 25-30%GC). Species fall into 4 groups on the basis of their 16s RNA catalogues. There are more than 100 species and as for *Bacillus*, the present classification of *Clostridium* is recognised to be unsatisfactory.	Grows anaerobically and forms spores under anaerobic conditions. Sulphate not reduced. Grows axenically.
Desulfotomaculum	Once included in the genus *Clostridium*, It consists of 10 species, 4 being thermophilic and 6 mesophilic.	Differs from *Costridium* by not reducing sulphate to sulphide.
Oscillospira	The genus only contains one species, *O. guillermondii*.	Mycelium formed. The rods or filaments are >3.0 um in diameter.
Pasteuria	Organisms are cultured witha nematode host as they have not been grown in pure culture. The species have branching hyphae, the terminal parts of which enlarge to form a structure similar to that of a sporangium in which ellipsoidal to spherical endospores are formed. Four species are recognised.	Mycelium formed. Requires an invertebrate host for growth.
Sporohalobacter	The genus is related to Halobacteroides and Haloanaerobium by similarity of their 16S RNA catalogues. Two species are represented by only one strain *S. lortetii*.	Halophilic anaerobe. Requires 0.5 mol l NaCl for growth. Gram -ve type cell wall.
Sporolactobacillus	Genus is represented by a single species *S. inulinus*. Resembles membes on the genus Lactobacillus , as it is a homolactic fermenter, does not have cytohromes , does not reduce nitrate and does not form indole.	Grows and forms spores under microaerophilic conditions. Catalase - ve.
Sporosarcina	Genus consists on 2 species-*S. ureae* and *S. halophila*.	Spore-forming aerobic cocci (in packets).
Sulfobacillus	Genus contains one species which resembles *Thiobacillus ferroxidans* in its substrates (ferrous iron, sulphide and sulphur) for energy metabolism and bieng acidophilic.	Aerobic and facultative. Non-sporing under anaerobic conditions. Catalase +ve. Growth at 45°C and pH 3.0. Ferrous iron, sulphide, and sulphur used as energy source.
Syntrophospora	Once classified as *Clostridium bryantii* and grows only in syntrophic coculture with *Methanospirillum hungatei*.	Differs from Clostridium by its requiremnt fora syntrophic coculture
Thermoactinomyves	The genus contains 7 species.	Mycelium formed. Grows axenically.

The information presented in Table 5.1 was based on information by Boeye & Aerts (1976), Logan & Berkeley (1981), Gordon (1981), Sneath (1986), Priest *et al.* (1987 & 1988), Parry *et al.* (1988), Koneman *et al.* (1992) and Berkeley & Ali (1994) presented in section 2.2. Examination of this table revealed that possibly 2 of the 13 genera (*Alicyclobacillus* and *Sulfobacillus*), were closely relate to the rod shaped, endospore-forming bacteria of the genus *Bacillus*. The latter genus exhibiting similar properties, being aerobic or facultative anaerobic, non-sporing under anaerobic conditions and producing catalase, however differentiation can be achieved by virtue of *Bacillus*'s inability to grow in a substrate (*p*H 3.0) at 45°C.

Prior to employing certain key morphological and/or physiological properties (which differentiated *Bacillus* species from one another), it was important to establish that the organism belonged to the genus *Bacillus* by identifying a few critical characteristics which pertains to members of this genus. These characteristics include: rod-shaped cells; endospores formed which are very resistant to many adverse conditions and with not more than one endospore per cell; sporulation was not repressed by exposure to air; Gram reaction positive: motility if present was by means of peritrichous flagellation: cells were either aerobic or facultatively anaerobic; colony morphology and size was *very variable* and some species produced pigments on certain culture media. Indeed, members of the genus *Bacillus* exhibited, acidophilic to alkaliphilic, some species were salt tolerant and others had specific requirements for salts (section 2.2).

This study employed the traditional approach to *Bacillus* identification, where the presumptive *Bacillus* isolate was assigned to one of three morphological groupings based on its endospore shape, relative size of the sporangium and whether or not its endospore caused the sporangium to distend. Morphological, biochemical and physiological tests were then used (presented in polythetic tables and/or dichotomous keys) to identify the organism to its species level. While a large number of species within the genus *Bacillus* have been traditionally arranged in these three morphological groups according to their endospore morphology, this probationary rule does not apply to *Bacillus* in the fourth unassigned grouping *"incertae sedis"*.

Figure 5.1 Flow chart for the identification of *Bacillus* spp.



Key morphological and physiological properties relating to the first three morphological groupings are presented in Figure 5.1, which is a diagrammatic flow scheme for the tentative identification of *Bacillus* to species level. This figure has been derived from the information supplied in section 2.2 and from subsequent experimental studies. Application of this flow scheme was based on identifying critical morphological and/or physiological tests, the result of each test (based on a positive or negative reaction) would determine the next test to be used. Identification was achieved when the terminus of a specific sequence of tests (governed by this flow scheme) resulted in a *Bacillus* species being tentatively nominated as the likely organism. An alternative 'numerical sequence' of tests could be employed to arrive at the same species identification (i.e. Figure 5.2, which is simply a rearrangement of the information presented in Figure 5.1).

Moreover, information supplied from Figure 5.1 (or Figure 5.2), supplemented with information presented in 1 of 3 tables (Table 5.2 to 5.4) which illustrates a detailed list of morphological and physiological properties associated with the various *Bacillus* species residing in the first 3 morphological groupings. Where Figure 5.1 helps differentiate between species by the application of a concise sequence of critical tests, Tables 5.2, 5.3 or 5.4 distinguish between closely related species by identifying a set of unique characteristics from the list of morphological/physiological properties which are considered to be representative of that particular species.

Morphological group one species are presently differentiated from the other morphological groups on the basis of the certain characteristics, namely; endospores are generally ellipsoidal or cylindrical in shape and do not appreciably distend the sporangium. The endospores have a central to subterminal position within the sporangium. *Bacillus megaterium* and members of the *B. cereus group (i.e. B. cereus*, *B. anthracis*, *B. mycoides*. and *B. thuringiensis*) have a larger cell width than the other species in group one (Table 5.2).

The cells of *B. megaterium* and *B. cereus* grown on glucose agar for 24 hours are filled with unstained globules, whereas the cells of the other species are not. Properties such as anaerobic growth, lecithovitellin/lecithinase reaction (section 3.5.20), acid production from mannitol (section 3.5.8.4), nitrate reduction (section

3.5.8.4), Voges-Proskauer reaction (section 3.5.8.4), and growth in lysozyme (section 3.5.19) were employed to differentiate between members of the *B. cereus* group and *B. megaterium* (Figure 5.1 and Table 5.2).

Figure 5.2 Tentative identification of typical strains of *Bacillus* spp.

Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by the *Bacillus spp.* name

1. Large Gram positive/ variable rod: positive → 2
2. Catalase: positive → 3.
   negative → 31.
3. Swelling of sporangium by ellipsoidal shaped spore: positive → 18.
   negative → 4.
4. Lecithovitellin/Lecithinase production: positive → 5.
   negative → 8.
5. Motility: positive → 6.
   negative → 7.
6. Parasporal body production: positive ⇒ ***B. thuringiensis***
   negative ⇒ ***B. cereus***
7. Haemovsis: positive ⇒ ***B. mycoides***
   negative ⇒ ***B. anthracis***
8. Reduction of nitrate (NO_3-) to nitrite (NO_2): positive → 9.
   negative →15.
9. Voges-Proskaeur (V-P) reaction: positive → 10.
   negative → 13.
10. Citrate utilization: positive → 11.
    negative → 12.
11. Maltose fermentation: positive ⇒ ***B. licheniformis***
    negative ⇒ ***B. subtilis***
12. Gelatin hydrolysis and growth in 7% sodium chloride: ⇒ ***B. coagulans***
13. Citrate utilization: positive ⇒ ***B. megaterium***
    negative → 14.
14. pH in V-P broth <5.7: ⇒ ***B. firmus***
15. Indole production: negative → 16.
16. Voges-Proskaeur (V-P) reaction: positive→ 17.
17. Starch hydrolysis: positive ⇒ ***B. coagulans***
    negative ⇒ ***B. pumilus***
18. Gas from glucose(inorganic N_2): positive →19.
    negative →22.
19. V-P reaction: positive → 20.
    negative → 21.
20. Production of dihydroxyacetone from glycerol: positive ⇒ ***B. polymyxa***
21. Production of dihydroxyacetone from glycerol: negative ⇒ ***B. macerans***
22. Starch hydrolysis: positive → 23.
    negative → 25.
23. Indole production: positive ⇒ ***B. alvei***
    negative → 24.
24. Growth at 65°C: positive ⇒ ***B. stearothermophilus***
    negative → ***B. circulans***
25. Catalase: positive → 26.
    negative → 28.
26. Survives serial transfers in Nutrient Broth: → 27.
27. Growth in anaerobic agar: positive ⇒ ***B. laterosporus***
    negative ⇒ ***B. brevis***
28. Fails serial transfers in Nutrient Broth: positive → 29.
29. Reduction of nitrate (NO3-) to Nitrite (NO2): positive ⇒ ***B. larvae***
    negative → 30.
30. Growth in 20% sodium chloride: positive ⇒ ***B. popilae***
    negative ⇒ ***B. lentimorbus***
31. Growth at 65°C: positive ⇒ ***B. stearothermophilus***
    negative → 28.
32. Swelling of the sporangium by spherical shaped spores: positive → 33.
33. Starch hydrolysis: negative → 34.
34. Urea or alkaline pH required for growth: positive ⇒ ***B. pasteurii***
    negative ⇒ ***B. sphaericus***

Members of the *B. cereus* group were distinguished from one another by means of differences in either motility, haemolysis production, non-rhizoid growth and/or inability to form parasporal bodies. The morphological and physiological/biochemical tests illustrated in Table 5.2 were applied to closely related species within morphological group one and the presumptive identification was confirmed by use of the API 20 E and API 50 CHB biochemical reaction profiles (section 3.8.5.4).

Other *Bacillus spp.* designated as belonging to morphological group once include: *Bacillus subtilis*, *B. licheniformis* and *B. pumilus* (these organisms share many morphological and physiological properties and collectively form the *B. subtilis* group) and differ from the *B. cereus* group on the basis that the cells within the former group have a smaller length and diameter (Table 5.2). Although *B. amyloliquefaciens* (presently placed in *incertae sedis*) does not presently reside among species classified under group one, its close affinity with *B. subtilis* has long been recognised and the organism has historically been given subspecies status as *B. subtilis subsp. amyloliquefaciens* (section 2.2).

Indeed, *B. amyloliquefaciens* is so closely related to each member of the *B. subtilis* group that it is extremely difficult to separate these organisms solely on the basis of classical tests and it was for this reason that *B. amyloliquefaciens* was not included as a separate species on the approved list of bacterial names (Sherman *et al.* 1980).

However, since 1980 a large body of evidence based on: differences in DNA homology studies (where DNA from strains of *B. amyloliquefaciens* has consistently been found to share less than 25, 13 and 5% homology with DNA sequences of *B. subtilis*, *B. licheniformis* an *B. pumilus* respectively); probabilistic identification methods based on API tests and also differentiation by pryolysis gas-liquid chromatography and pyrolysis mass spectrometry now suggest that *B. amyloliquefaciens* can be separated from members of the *B. subtilis* group (section 2.2). Some of the more important biochemical, physiological and morphological characteristics which distinguishes *B. amyloliquefaciens* from *B. subtilis* and other closely related *Bacillus* species are illustrated in Table 5.3.

Table 5.2 Key Morphological and biochemical properties of *Bacillus spp.* assigned to morphological group 1.

BIOCHEMICAL AND CELL CHARACTERISTICS (Morphological Group 1)	BACILLUS SPECIES												
	B. megaterium	*B. cereus I*	*B. cereus II*	*B. mycoides*	*B. anthracis*	*B. thuringiensis*	*B. licheniformis*	*B. subtilis*	*B. pumilus*	*B. amyliolique faciens*	*B. firmus*	*B. lentus*	*B. coagulans*
Gram Reaction	+	+	+	+	+	+	+	+	+	+	+	+	+
Chains of Cells	+	+	+	+	+	+	d	d	+	d	d	d	d
Cell Width (>1.0µm)	+	+	+	+	+	+	-	-	-	-	-	-	-
Cell Length (>3.0µm)	d	+	+	+	+	+	-	-	-	-	-	-	+
Spore Shape	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY	E/CY
Spore Position	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/TST
Swelling of Sporangium	-	-	-	-	-	-	-	-	-	-	-	-	V
Acid Production in (Litmus Milk)							-	-	-	-	-	-	+
Lecithinase Reaction	-	+	+	+	+	+	-	-	-	-	-	-	-
Anaerobic Growth	-	+	+	+	+	+	+	-	-	-	-	-	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	-	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	-	V
β-Haemolysis		+	+	+	-	+							
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 7% NaCl	+	+	+	+	+	+	+	+	+	+	+	-	-
Growth at 50°C	-	-	-	-	-	-	+	d	d	+	-	-	+
Growth at 55°C	-	-	-	-	-	-	+	-	-	-	-	-	+
Growth at 65°C	-	-	-	-	-	-	-	-	-	-	-	-	d
Motility	+	+	+	-	-	+	+	+	+	+	+	+	+
Growrh in 0.001% lysozyme	-	+	+	+	+	+	-	-/+	-/+		-		-
Parasporal Body	-	-	-	-	-	+	-	-	-		-		-
API20E Reaction Profile[1]													
ONPG	83	0	0	43	0		100	95	100	85	21	95	100
ADH	0	85	2	56	0		93	5	4	0	7	5	0
Citrate Utilization	77	78	99	93	0		77	89	86	75	100	66	0
Urease Activity	0	0	0	18	0		3	5	0	0	0	5	0
Indole Production	0	0	0	0	0		0	0	0	0	0	0	0
Voges-Proskaeur Reaction	88	60	99	100	100		80	100	100	50	71	33	100
Gelatin Hydrolysis	77	100	100	100	100		87	94	25	95	50	0	50
Nitrate(NO_3-) Reduction	77	78	85	43	100		87	94	25	95	50	0	50
LDC	0	0	0	0	0		0	0	0	0	0	0	0
API50CHB Profile[1] ((Acid profilre from 49 Ammonium Salt Sugars)													
Glucose (No.11)	83	100	100	100	100		83	100	100	100	70	100	100
Mannitol (No.18)	66	0	0	6	0		0	5	0	0	0	0	0
Xylose (No.7)	0	0	0	0	0		0	0	0	0	0	0	0
Arabinose (No.4)	83	0	0	6	0		100	94	90	90	75	0	0

Table 5.3 Key morphological and biochemical characteristics which differentiate members of the *B. subtilis* subgroup in morphological group 1.

Characteristics	% Of Strains positive			
	B. amyloliquefaciens[1]	B. subtilis	B. licheniformis	B. pumilus
API tests *Acid produced from:*				
Galactose	44	30	100	100
Sorbitol	88	88	95	17
Inulin	11	83	68	1
Arginine dihydrolysis	0	0	95	0
α-L-Arabinosidase	0	75	100	100
N-benzoyl-L-leucine aminopeptidase	0	0	100	12
α-D-Glucosidase	12	100	100	0
β-D-Glucosidase	0	87	75	100
L-Pyrrolidone aminopeptidase	100	100	100	
L-Tryptophan aminopeptidase	100	0	100	87
Degradation tests:				
DNA	33	93	100	100
$NO_3 \rightarrow NO_2$	78	100	100	0
Starch	100	100	100	0
Pectin	0	27	95	85
Physiological tests:				
Anaerobic growth	0	0	100	0
Phosphatase	100	67	100	15
Propionate utilization	0	0	100	0
Chain formation	84	22	80	5
Guanine-plus-cytosine content (mol%)	44-46	42-48	43-47	42-47

[1] The type strain conforms to the pattern of characteristics of the majority of the strains.

Due to the very close similarity between *B. amyloliquefaciens* and the members of the *B. subtilis* group, the former organism was identified along with this grouping for practical identification purposes. Information shown in Tables 5.2 and 5.3 permitted the differentiation between species in the *B. subtilis* group (which otherwise were very closely related).

Indeed, *Bacillus licheniformis* was tentatively separated from the other species in the *B. subtilis* group largely on the grounds of its chitin decomposition, anaerobic production of gas from nitrate, arginine hydrolysis, nitrate reduction and acetate/tartrate utilization (Table 5.3). Indeed the API biochemical galleries have proved extremely valuable in differentiating between *B. licheniformis* and *B. subtilis* (*e.g.* variation between species on the ability to produce acid from galactose, inulin and dihydrolysis of arginine). *Bacillus pumilus* differs from the *B. subtilis* group by its inability to hydrolyse starch and reduce nitrate. For all species residing within this *B. subtilis* group, unstained globules are not demonstrated in the protoplasm of slightly stained cells on glucose agar.

Reference to 'The Colour Atlas of *Bacillus Species*' (Perry *et al* 1992) provided a useful guide in the idenification of presumptive *Bacillus* species which exhibited a characteristic surface culture appearance when cultivated on Blood Agar plates. However, extreme caution was taken when identifying each *Bacillus* isolate on the basis of its surface culture appearance, as different species often exhibited a similar colony morphology and strains of the same species were shown to vary considerably in this characteristic.

Although quite similar in morphological appearance to the *B. subtilis* group, strains of *B. firmus* are distinguishable from this group by having a negative Voges-Proskaeur reaction, inability to utilise citrate and a sensitivity to acid (i.e. cultures of *B. firmus* form acid from glucose but were so sensitive to acids that growth ceases at pH 6.0 or above). Although *B. lentus* is an unassigned species, (presently tentatively positioned in subgroup B of *incertae sedis*), there is a strong association between some strains and that of the species *B. firmus* , where they differ by not decomposing casein and gelatin and the inability to produce urease (Table 5.3).

In contrast to *B. firmus*, strains of *B. coagulans* are aciduric and produce a low pH (4.0-4.5) in media containing utilizable carbohydrates (Figure 5.1). Strains of *B. coagulans* are morphologically variable. The rods of some strains resemble those of the *B. subtilis* group, and differ largely on the grounds that the cells are longer and slender compared to *B. coagulans* and the sporangium may or may not swell around the spore. Therefore it would appear that *B. coagulans* is intermediate between group 1 and 2, as the main criterion which distinguishes both groups of species is whether or not the endospore distends the sporangium. Strains of *B. coagulans* may vary in nutritional requirements and can be separated into 2 types. Type A have a negative Voges-Proskaeur reaction and grow at 65°C whereas type B are the opposite, having a positive Voges-Proskaeur reaction and not being able to grow at 65°C.

Morphological group two species are characterised by swelling of the sporangia by the ellipsoidal shaped endospore; spores are located centrally or subterminally within the sporangium, Gram reaction is positive to variable. The application of the information supplied in Figure 5.1 (or Figure 5.2) and Table 5.4 permits identification of these *Bacillus* to species level. The key distinguishing morphological and/or biochemical characteristics of morphological group two species include: *B. polymyxa* (does not form indole, hydrolyses starch, forms gas form carbohydrates, produces acetone, exhibits a ribbed spore surface and produces dihydroxyacetone from glycerol); *B. macerans* (does not form indole, acetone not produced, dihydroxyacetone from glycerol is negative, produces crystalline dextrin); *B. circulans* (indole not formed, non gas formation from carbohydrates, non production of crystalline dextrins, motile colonies); *B. stearothermophilus* (growth at 65°C, indole not formed, some strains are catalase negative); *B. alvei* (indole formed, motile colonies on agar surface); *B. laterosporus* (canoe shaped parasporal body attached to spore, starch not hydrolysed, facultative anaerobe, pH <8.0 in glucose broth after culture); *B. brevis* (starch not hydrolysed, aerobe, pH ≥8.0 in glucose broth after culture (ammonia produced); *B. larvae* (starch not hydrolysed, catalase negative, casein decomposed, nitrate reduced to nitrite, fails serial transfers in nutrient broth);

B. popillae {nitrate not reduced, casein decomposed, growth in 2% NaCl); *B. lentimorbus* {nitrate not reduced, casein decomposed, no growth in 2% NaCl).

Table 5.4 Key morphological and biochemical properties of *Bacillus* assigned to morphological group 2.

	BACILLUS SPECIES									
BIOCHEMICAL AND CELL CHARACTERISTICS (Morphological Group 2)	***B. polymyxa***	***B. macerans***	***B. circulans***	***B. stearothermophilus***	***B. alvei***	***B. laterosporus***	***B. brevis***	***B. larvae***	***B. popillae***	***B. lentimorbus***
Gram Reaction	+-v	+-v	+-v	+-v	+-v	+-v	+-v	+-v	+-v	+-v
Chains of Cells	-	-	d	d	d	-	-			
Cell Width (>1.0μm)	-	-	-	-	-	-	-	-	-	-
Cell Length (>3.0μm)	d	d	d	-	+	d	d	-	-	-
Spore Shape	E	E	E	E	E	E	E	E	E	E
Spore Position	C/ ST	C/ ST	C/ T/ ST	C/ ST	C/ ST	C/ ST	C/ ST			
Swelling of Sporangium	+	+	+	+	+	+	+	+	+	+
Lecithinase Reaction	-	-	-	-	-	+	-	-	-	-
Anaerobic Growth	+	+	v	-	+	+	-		+	
Starch Hydrolysis	+	+	+	+	+	-	-	-	-	-
Casein Hydrolysis	+	-	d	-	+	+	+	+	-	-
Catalase	+	+	+	d	+	+	+	-	-	-
Growth in 7% NaCl	-	-	v	-	-	-	-	-	+	-
Growth at 50°C	-	d	d	+	-	+	+			
Growth at 55°C	-	-	-	+	-	-	-			
Growth at 65°C	-	-	-	+	-	-	-			
Motility	+	+	+	+	+	+	+			
C-Shaped Parasporal body	-	-	-	-	-	+	-	-	-	-
Gas from Carbohydrates	+	+	-	-	-	-	-	-	-	-
Dihygroxyacetone from glycerol	+	-								
pH in Glucose Broth(>8)	-	-	-	-	-	-	+			
Survives serial transfers in Nutrient Broth	+	+	+	+	+	+	+			
API20E Reaction Profile										
ONPG	100	100	85	10	80	0	25			
ADH	0	26	1	0	0	0	5			
Citrate Utilization	52	30	42	0	0	75	75			
Urease Activity	0	0	1	0	75	0	3			
Indole Production	0	0	1	0	50	50	0			
Voges-Proskaeur Reaction	90	73	57	12	100	50	57			
Gelatin Hydrolysis	95	50	14	93	100	100	40			
Nitrate(NO_3-) Reduction	71	36	42	85	25	75	50			
LDC	0	0	0	0	0	0	0			
API50CHB Profile (Acid profile from 49 Ammonium Salt Sugars)										
Glucose (No.11)	100	100	100	100	100	100	15			
Mannitol (No.18)	100	10	99	31	0	75	18			
Xylose (No.7)	0	5	0	0	0	0	0			
Arabinose (No.4)	100	100	99	25	0	0	0			

Of the starch hydrolysing species within group two, *B. polymyxa* is the most easily recognised due to its endospores demonstrating a heavily ribbed surface. Although *B. macerans* resembles *B. polymyxa* in its spore morphology and its active formation of gas from carbohydrates, the former can be differentiated by not producing acetone and dihydroxyacetone from glycerol. At present only gas production from carbohydrates and formation of crystalline dextrins differentiates *B. macerans* from *B. circulans* (Table 5.4). Indeed, *B. circulans* encompasses a group of morphologically, nutritionally , physiologically and chemically heterogeneous strains.

The thermophile *B. stearothermophilus*, is also considered heterogeneous. The spores formed by a culture of this thermophile vary in size. Growth at 65°C differentiates *B stearothermophilus* from *B. circulans* and some strains of the former species are known to be catalase negative (Figure 5.1).

Bacillus alvei , like *B. circulans* may form motile colonies on the agar surface. But unlike *B. circulans*, it degrades tryptophane and indole is also formed. The facultative anaerobe *B. laterosporus* has a distinct canoe-shaped parasporal body attached to the spore; this renders the spore in a lateral position within the sporangium. Unlike most of the other *Bacillus* species in group two, *B. laterostorus* does not hydrolyse starch. The ellipsoidal spores of *B. brevis* are centrally located and distend the sporangia into spindle shapes. *B. brevis* resembles strains of *B. laterosporus* in that it cannot hydrolyse starch, but the former is easily differentiated from *B. laterosporus* by being aerobic and cultures growing in glucose broth have a pH >8.0 or higher due to the strong production of ammonia from organic nitrogen (Figure 5.1).

Strains of *B. larvae*, *B. popillae* and *B. lentimorbus* are catalase negative, grow slowly and require special media for growth. Properties such as nitrate reduction, motility , casein hydrolysis and growth in 2% NaCl separates these three species.

Morphological group three species are characterised by swelling of the sporangium with spherical shaped spores which are located centrally to subterminally. The flow scheme illustrated in Figure 5.1 describes how to go about identifying both *B. sphaericus* and *B. pasteurii* which comprise morphological group 3. Although *B. sphaericus* possesses very similar physiological, nutritional and morphological properties to that of *B. laterosporus* and *B. brevis*, the former species can be

separated by its spherical spore shape, deamination of phenylalanine, non-decomposition of tyrosine and casein and non-reduction of nitrate to nitrite. The application of API 20 E/ 50 CHB reaction profiles has proved to be a useful tool in differentiating between these three closely related species. *B. pasteurii* requires special conditions for growth, such as alkaline media containing ammonia and urea (Figure 5.1).

The remainder of the *Bacillus* species are unassigned (i.e. the number of available representative strains is insufficient to establish a stable, reliable characteristic) and therefore grouped under the collective heading of *"incertae sedis"*. In other instances, time had not permitted a comparison of the representative strains with a significant number of other *Bacillus* species (section 2.2).

Subgroup A include anaerobic species which hydrolyse starch/casein and gelatin, produce oval shaped spores, and fail to grow at 3°C. This subgroup includes: *B. apiarus* (citrate utilized), *B. filicolonicus* (acid from mannitol), *B. thiaminolyticus* (indole produced) and *B. alcalophilus* (non acid from glucose).

Subgroup B include aerobic species which hydrolyse starch, produce oval shaped spores and fail to grow at 3°C. This subgroup includes: *B. cirroflagellosus* (nitrate reduced), *B. chitinosporus* (casein and gelatin hydrolysed) and *B. lentus* (urease activity).

Although *Subgroup C* include aerobic species that produce oval shaped spores and fail to grow at 3°C, they differ from subgroup B by not being able to hydrolyse starch. Subgroup C includes: *B. badius* (casein and gelatin hydrolysed), *B. aneurinolyticus* (nitrate reduced), *B. macroides* (gelatin hydrolysed) and *B. freundenreichii* (urease activity).

Subgroup D comprises of both anaerobic and aerobic species which produce oval to spherical spores, hydrolyse starch and fail to grow at 3°C. This subgroup include: *B. pantotheniticus* (anaerobe and similar to *B. coagulans* and *B. stearothermophilus)* and *B. epiphylus* (aerobe).

Subgroup E_1 consist of aerobic species which produce spherical spores, hydrolyse starch and grow at 3-5°C. This subgroup includes: *B. aminovorans, B. globisporus*

(psychrophile and nearest relative to *B. sphaericus*) *B. insolitus* and *B. psychrophilus* (psychrophile and close relative to *B. sphaericus*).

Subgroup E_2 consists of aerobic species which hydrolyse starch, form oval spores and grows at 3°C. This subgroup includes: *B. psychrosaccharolyticus* (pleomorphic psychrophile -coccal to elongate) and *B. macquariensis*.

In addition to presumptively identifying an unknown surface culture to *Bacillus* species level (by way of following the morphological and/or biochemical tests outlined in the identification flow scheme in Figure 5.1 or 5.2 and by matching these characteristic properties with those shown Tables 5.3 to 5.4), confirmation of this tentative *Bacillus species* identification was achieved by carrying out an API 20 E and API 50 CHB biochemical profile analysis (section 3.5.8.4). The resulting API profiles being interrupted by keying both sets of sequences into the APIlab Plus computer software package (section 3.5.8.5).

In order to validate the above identification scheme, 12 different *Bacillus* species (obtained from either National Collection of Typed Cultures, Colindale, London or from the National Collections of Industrial and Marine Bacteria Ltd, Aberdeen, Scotland) were identified. They included: *B. licheniformis* (NCTC 10341), *B. subtilis* (NCTC 3610), *B. amyloliquefaciens* (NCIMB 10785), *B. pumilus* (NCTC 10337), *B. cereus* type I (NCTC 11143), *B. cereus* type II (NCTC 11145), *B. mycoides* (NCTC 926), *B. brevis* (NCTC 2611), *B. laterosporus* (NCTC 7579), *B. lentus* (NCTC 4824), *B. sphaericus* (NCTC 7582) and *B. circulans* (NCTC 9432).

The results of this validation study revealed that application of the tests outlined in flow scheme (Figure 5.1 or 5.2) and from the relevant tables (Tables 5.2 to 5.4) did correctly identify the presumptive *Bacillus* strains to species level. The results of the API confirmatory tests are illustrated in the appendix (Tables A53 to A62). However, many of the biochemical tests (e.g. starch hydrolysis) which were considered characteristic of the test organism, proved to be either negative or extremely vague (i.e. weak reaction which was often difficult to observe), and application of the API galleries were often necessary for a confident identification to species level.

5.2 The microbiological quality of infant milk formulae.

The purpose of this study was to examine 100 reconstituted infant milk formulae (purchased in Scotland over a 12 month period) in order to determine the effects of various methods of preparation, cooling and storage on either the type and number of *Bacillus* spp. present and/or the detection of heat damaged *Listeria* cells which may have contaminated the powder. Where *Bacillus cereus* type II was isolated, an additional assay for diarrhoegenic enterotoxin production was performed using the *Bacillus cereus* Reverse Phase Latex Agglutination test (section 3.9).

Each month 8 to 10 infant milk formulae (IMF) products representative of the main brands available in the UK (viz. Cow & Gate Ltd., John Wyeth Ltd., Farleys Health Products Ltd., and Milupa Ltd.) were purchased and analysed. Duplicate samples of IMF were reconstituted at a water temperature of 56°C and/or 90°C according to instructions described in section 5.1(i.e. using sterile water and aseptic techniques in order to avoid introducing microorganism from the environment), and pasteurised and/or unpasteurised samples were subjected to a range of cooling and storage procedures commonly used in homes and/or in hospitals.

Cooling procedures included: initial table top cooling followed by refrigeration, immediate refrigeration, and initial water cooling followed by refrigeration. Refrigeration temperatures of both 4°C and 10°C were used since it is well known that many refrigerators (both at home and in hospitals) operate at temperatures well in excess of the recommended 4°C (section 2.3.2). In addition to the experiments described above, samples were also subjected to the "temperature abuse" that may occur when these products are used or are inadequately stored either in the home or hospitals after reconstitution. Following a period of refrigeration the reconstituted formulae was stored at either 10°C, 20°C, 25°C, 30°C and/or 35°C for up to 24 hours to simulate the temperatures that may be encountered in hospital wards and incubators for premature infants.

Infant formula samples were also pasteurised after reconstitution and then subjected to the procedures described above. Samples were taken for microbiological analysis at regular intervals (as described in section 5.1) and a record of the temperature profile of the products was kept. In addition, samples were analysed for the presence

of heat stressed *Listeria* cells via the modified United States Department of Agriculture (USDA) and the modified Food and Drugs Administration (FDA) methods (Table 5.5). Conditions previously shown to enhance the recovery of damaged *Listeria* cells were employed (section 4), i.e. samples were reconstituted at 56°C and enriched for 14 hours in a stationary position (0 rpm) at 30°C prior to transfer to the FDA and/or USDA selective enrichment broths. At the end of each enrichment stage, samples were streak plated onto TSYEA and LSA (Oxford formulation) where they were incubated for 72 hours at 37°C prior to screening for atypical and typical *Listeria* survivors.

Table 5.5 Recovery of heat damaged *L. monocytogenes* using the modified "FDA" and modified "USDA" methods.

Procedure	Modified FDA	Modified USDA
Pre-enrichment	Reconstitute IMF at 56°C and store for 14 h at 30°C[†].	Reconstitute IMF at 56°C and store for 14 h at 30°C[†].
Primary enrichment	Add 5 ml to 25 ml of EB and store for 24 and 48 h at 30°C.	Add 5 ml to 25 ml of LEB and store for 24 h at 30°C.
Secondary enrichment	Add 0.1 ml to 9.9 ml Modified FB and store at 35°C for 24 and 48 h.	Add 0.1 ml to 9.9 ml Modified FB and store at 35°C for 24 and 48 h.
Plating media	Loop inoculate TSYEA and LSA plates after each primary and secondary enrichment stage. Store plates for 24, 48 and 72 h at 35°C.	Loop inoculate TSYEA and LSA plates after each primary and secondary enrichment stage. Store plates for 24, 48 and 72 h at 35°C.
Confirmation tests	CAMP, Catalase, Motility, Gram stain, Henry Illumination, API Listeria and API MICRO ID.	CAMP, Catalase, Motility, Gram stain, Henry Illumination, API Listeria and API MICRO ID.

[†] 30 g IMF reconstituted in 220 ml water as described in section 5.1
Abbreviations: EB, Enrichment broth; LEB, Listeria enrichment broth (University of Vermont formulation); FB, Fraser broths; TSEYA, Tryptone sova agar supplemented with 0.6% yeast extract; LSA, Listeria selective agar (Oxford formulation).

In order to examine growth of *Bacillus* spp. and/or *Listeria* spp. under the different preparation, cooling and storage conditions described earlier, it was necessary to carry out a series of separate experiments (Table 5. 6). This table shows the variety of cultural parameters investigated over the course of the 12 month study.

According to the International Commission of Microbiological Safety of Foods (section 2.3.2), all 100 IMF units analysed prior to subsequent storage were of satisfactory microbiological quality as their Total Aerobic Mesophilic Counts (CFU g^{-1}) did not exceed the "satisfactory" threshold value of 10^4 CFU g^{-1} IMF. Indeed,

Table 5.6 Combination of preparation (reconstitution temperature, pasteurisation and cooling method) and storage conditions employed over the course of the study

No. of IMF	Reconstitution Temp (°C)		Cooling Method			Pasteurized (63°C/30 mins)		Storage Temperature (°C)					
Sampled	56	90	Tap	Table Top	Ref‡	No	Yes	35	30	25	20	10	4
24	+	+	+	+	+	+	-	+	-	+	-	+	+
8	+	+	+	+	+	+	+	+	-	+	+	+	+
6	+	+	+	+	-	+	+	+	+	+	+	+	+
7	+	+	+	-	-	+	+	-	+	+	-	+	+
6	+	+	+	+	-	+	+	-	-	+	-	-	-
6	+	+	+	+	-	+	-	-	+	+	-	+	+
5	+	+	+	+	-	+	-	-	-	+	-	+	+
6	+	+	-	+	-	+	-	-	-	+	-	+	-
4	+	+	+	-	-	+	-	-	-	+	-	+	-
8	+	+	+	-	-	+	+	-	-	+	-	+	-
3	+	+	+	+	+	+	+	-	+	+	-	-	+
6	+	-	+	+	-	+	-	-	+	-	-	-	-
2	+	+	+	+	-	+	-	-	+	-	-	-	-
3	+	+	+	+	-	+	+	-	-	+	-	-	+
2	+	+	+	+	-	+	+	-	-	+	-	+	+
2	+	+	+	-	-	+	-	+	-	+	+	-	+
1	+	+	+	+	-	+	-	-	+	-	-	-	+
1	+	-	-	+	-	+	+	-	-	+	-	-	+
Total 100	**100**	**93**	**93**	**79**	**35**	**100**	**44**	**40**	**30**	**91**	**16**	**68**	**68**

‡ IMF samples refrigerated after reconstitution at 56 and/or 90°C
† IMF samples analyzed at 0 and 8 hours in addition to 14 and 24 hours illustrated above

Table 5.7 Total Aerobic Mesophilic Count (CFU g^{-1}) obtained for the 100 reconstituted infant milk formulae after reconstitution and prior to storage.

IMF Product	Total No. of samples (n)	Number of IMF samples having the following Total Aerobic Mesophilic Counts at 0 hr (cfu g^{-1}) at the reconstitution temperatures of 56 and 90°C												Total Aerobic Mesophilic Count (cfu g-1) at reconstitution temperature of 56°C and 90°C					
		≤ $1.0x10^{2}$		≤ $5.0x10^{2}$		≤ $1.0x10^{3}$		≤ $5.0x10^{3}$		≤ $1.0x10^{4}$		> $1.0x10^{4}$		Mean		S.D.		Range	
		56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°	56°C	90°C	56°C	90°C	56°C	90°C
SMA White Cap	13	8	6	2	5	2	1	1	1	0	0	0	0	$3.2x10^{2}$	$6.9x10^{2}$	$6.0x10^{2}$	$6.1x10^{2}$	$NDx10^{2}$ $2.1x10^{3}$	$NDx10^{2}$ $2.3x10^{3}$
SMA Gold Cap	12	6	3	5	8	1	1	0	0	0	0	0	0	$2.7x10^{2}$	$3.0x10^{2}$	$2.2x10^{2}$	$2.4x10^{2}$	$NDx10^{2}$ $6.0x10^{2}$	$NDx10^{2}$ $9.0x10^{2}$
Milupa Aptamil	12	3	6	6	4	1	0	1	1	1	1	0	0	$8.3x10^{2}$	$7.5x10^{2}$	$1.9x10^{3}$	$1.6x10^{3}$	$NDx10^{2}$ $6.1x10^{3}$	$NDx10^{2}$ 5.6x103
Milupa Milumil	11	5	5	2	2	1	3	3	1	0	0	0	0	$6.7x10^{2}$	$6.2x10^{2}$	$1.0x10^{3}$	$1.0x10^{3}$	$NDx10^{2}$ $3.5x10^{3}$	$NDx10^{2}$ $3.6x10^{3}$
Nutrilon Premium	14	7	9	7	4	0	1	0	0	0	0	0	0	$1.4x10^{2}$	$1.4x10^{2}$	$1.0x10^{2}$	$1.7x10^{2}$	$NDx10^{2}$	$3.0x10^{2}$
Nutrilon Plus	11	6	7	5	4	0	0	0	0	0	0	0	0	$1.5x10^{2}$	$1.4x10^{2}$	$9.3x10^{1}$	$1.3x10^{2}$	$NDx10^{2}$ $3.0x10^{2}$	$NDx10^{2}$ $4.0x10^{2}$
Farleys Oster Milk One	11	8	9	3	2	0	0	0	0	0	0	0	0	$1.0x10^{2}$	$1.0x10^{2}$	$1.1x10^{2}$	$1.3x10^{2}$	$NDx10^{2}$ $3.0x10^{2}$	NDx102 4.0x102
Farleys Oster Milk Two	11	2	3	6	5	0	2	3	1	0	0	0	0	$5.9x10^{2}$	$4.5x10^{2}$	$6.2x10^{2}$	$4.8x10^{2}$	$NDx10^{2}$ $1.7x10^{3}$	$NDx10^{2}$ $1.6x10^{3}$
Farleys First Milk	3	2	1	1	2	0	0	0	0	0	0	0	0	$1.3x10^{2}$	$1.6x10^{2}$	$1.5x10^{2}$	$1.5x10^{2}$	$NDx10^{2}$ $3.0x10^{2}$	$NDx10^{2}$ $3.0x10^{2}$
Farleys Second Milk	2	1	0	1	2	0	0	0	0	0	0	0	0	$2.5x10^{2}$	$2.5x10^{2}$	$3.5x10^{2}$	$5.0x10^{1}$	$NDx10^{2}$ $5.0x10^{2}$	$2.0x10^{2}$ $3.0x10^{2}$
Total No. of IMF	**100**	**48**	**49**	**38**	**38**	**5**	**8**	**8**	**3**	**1**	**1**	**0**	**0**	**$3.4x10^{2}$**	**$3.3x10^{2}$**	**$2.6x10^{2}$**	**$2.2x10^{2}$**	**$NDx10^{2}$ $6.1x10^{3}$**	**$NDx10^{2}$ $5.6x10^{3}$**

Table 5.7 shows the variation in microbiological quality amongst the individual products with 48%, 38%, 5%, 8%, 1% and 0% of the microbial load falling within the range $\leq 1.0x10^2$, $\leq 5.0x10^2$, $\leq 1.0x10^3$, $\leq 5.0x10^3$, $\leq 1.0x10^4$ and $\geq 1.0x10^4$ CFU g^{-1} powder respectively. The mean Total Aerobic Mesophilic Count for samples reconstituted at a water temperature of 56°C and 90°C was $3.4x10^2$ and $3.3x10^2$ CFU g^{-1} powder respectively, while the maximum Total aerobic count achieved prior to storage was $6.1x10^3$ and $5.6x10^3$ CFU g^{-1} powder.

The temperature at which the IMF samples were reconstituted did not significantly effect the microbial content (P0.05). The variation in preparation and cooling conditions (prior to subsequent storage) did not significantly effect (P 0.05) the type and/or numbers of organism recovered. *Bacillus licheniformis* and *B. subtilis* were found to be the most frequently isolated organisms recovered from dried infant powder, being detected in 46% and 30% of all IMF sampled (Table 5.8). Other members of the genus *Bacillus* recovered without the requirement of further incubation include: *B. cereus I* (10%), *B. cereus II* (7%), *B. pumilus* (1%), *B. megaterium* (6%), *B. sphaericus* (8%), *B. amyloliquefaciens* (1%), *B. firmus* (4%), *B. coagulans* (6%), *B. circulans II* (4%), *B. brevis* (1%), *B. polymyxa* (3%) *and B. mycoides* (5%) (Table 5.8).

Listeria spp. (including *L. monocytogenes*) were not recovered from the dried infant powder via the modified FDA and/or USDA methods. Storage of reconstituted IMF for 8, 14 and 24 hours at temperatures ≥20°C resulted in a change in the type, number and concentration (CFU g^{-1}) of indigenous *Bacillus* spp. present (Table 5.8), with the emergence of 2 *Bacilli* (i.e. *B. lentus* and *B. laterosporus*) which were not recovered at the point of formula preparation. The effect of separate preparation, cooling and storage treatments are not illustrated in this table, rather it simply shows whether the organism was present at a particular sample time period under any combination of treatments.

The difference in frequency and type of organism recovered from the dried infant powder (0 hour storage) and that of the same IMF which had been reconstituted and stored for 24 hours at temperatures ≥25°C is illustrated in Figures 5.3(a) and 5.3(b) respectively. Examination of these figures revealed that storage of rehydrated infant

formulae resulted in either no change or an increase in the frequency of occurrence of different *Bacillus* spp. However, the variation in preparation and cooling conditions, in conjunction with additional storage of samples did not result in the recovery of thermally or osmotically damaged *Listeria* cells. Therefore, *Listeria* spp. were not isolated from 100 commercial IMF products during this study. With the exception of certain members of the *B. subtilis* group (i.e. *B. licheniformis* and *B. subtilis*) and *B. firmus*, the number of samples supporting the growth of different *Bacillus* spp. significantly increased at storage times ≥8 hours.

While *B. licheniformis* and *B. subtilis* were the most frequently isolated organisms recovered from IMF at 0 hours, subsequent storage revealed a significant increase in the emergence of *B. cereus* type *I* and *II* where these organisms were recovered from the all samples in similar numbers.

The isolation of *Bacillus* spp. from the 10 varieties of reconstituted IMF over the 24 hour storage period are shown in Tables 5.9 (a, b and c). Examination of these tables revealed that the different *Bacillus* spp. were recovered from a wide variety of IMF and that the microbiological quality (i.e. number of IMF supporting growth of a particular organism) exhibited by these IMF samples were not significantly different. Moreover, the tables show an even distribution of each type of *Bacillus* spp. among the different IMF products.

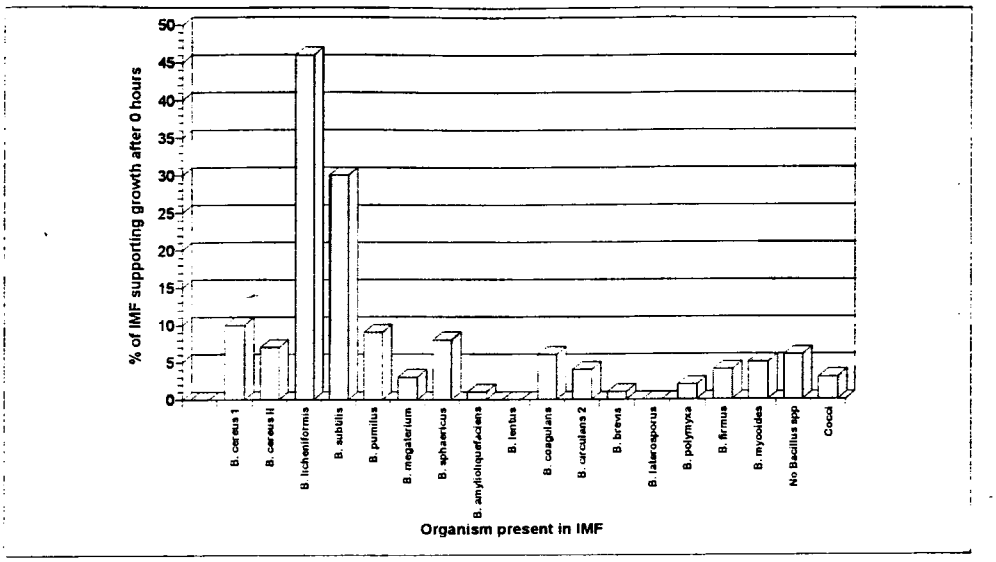
The variation in Total Aerobic Mesophilic Counts (CFU g^{-1}) exhibited by the IMF samples analysed under a particular combination of preparation (56°C and/or 90°C), cooling (tap cooled, table top cooled, and refrigerated) and storage conditions (≤10°C, 20°C, 25°C, 30°C and 35°C) is shown in Table 5.10. Indeed, the information presented in this table was derived from the results of 24 separate studies which have been illustrated in 50 tables (Tables 1-50 in appendix).

Examination of Table 5.10 (and Tables 1 to 50 in appendix) revealed that the temperature of reconstitution, method of cooling and pasteurisation of samples did not significantly influence the mean Total Aerobic Mesophilic Counts (CFU g^{-1}) obtained for the 100 IMF products analysed. However, other conditions such as, the temperature and duration of storage did significantly influence the type, number and concentration of *Bacillus* spp. present.

Table 5.8 Number of IMF samples from which different *Bacillus* spp. were isolated over a 24 hour storage period.

***Bacillus* Spp.**	**Number of IMF which the named *Bacillus* spp were detected over a 24 hour period.**			
	0 hours	**8 hours**	**14 hours**	**24 hours**
B. cereus I	10	30	31	31
B. cereus II	7	34	37	38
B. licheniformis	46	46	47	31
B. subtilis	30	36	36	32
B. pumilus	9	13	13	11
B. megaterium	3	12	13	13
B. sphaericus	8	20	21	21
B. amloliquefaciens	1	2	2	2
B. lentus	ND	3	3	3
B. coagulans	6	8	8	8
B. circulans 2	4	6	6	6
B. brevis	1	3	3	3
B. laterosporus	ND	ND	1	1
B. polymyxa	3	4	4	4
B. firmus	4	4	4	4
B. mycoides	5	12	13	13
No Bacillus spp. detected	6	2	1	6
Non Bacillus spp. (Cocci)	3	6	6	6
Listeria spp.	0	0	0	0

(a)



(b)

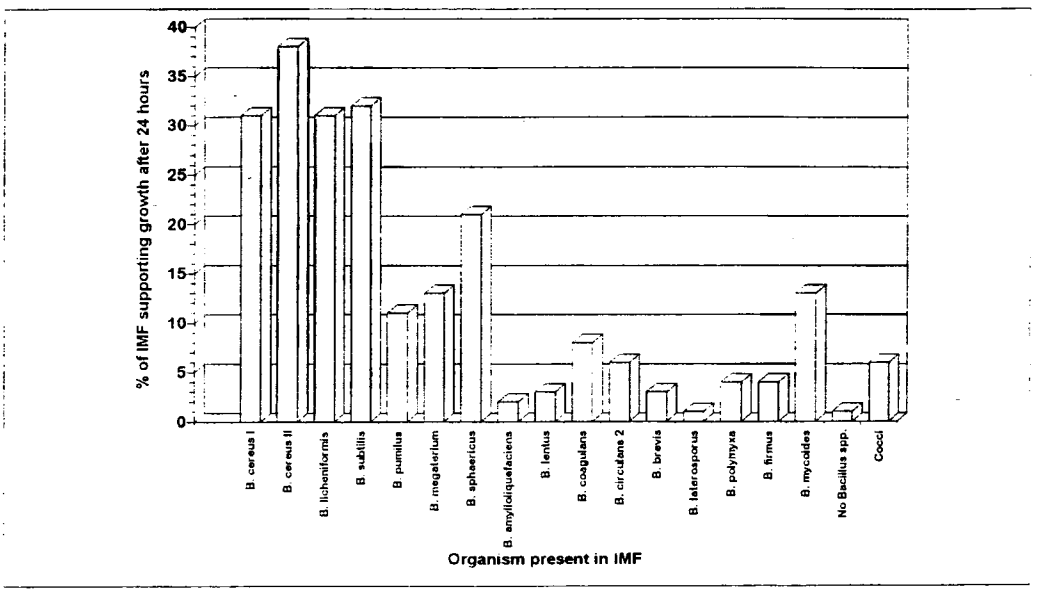


Figure 5.3 (a) and (b) illustrates the percentage of IMF samples from which different *Bacillus* spp. were isolated at 0 and 24 hours storage (≥25°C) respectively.

While the method of cooling did not dramatically effected the average number and concentration (CFU g^{-1}) of *Bacillus spp.* at a reconstitution temperature of 56°C, where a significant increase or decrease in Total Viable Counts occurred at the higher preparation temperature of 90°C, then the extension of heat exposure brought on by the table top cooling method did augment the particular trend observed. Immediate tap cooling and refrigeration of IMF prepared at 90°C did not effect the Total Aerobic Mesophilic Counts obtained. Preparation of IMF samples at this elevated temperature resulted in the emergence of certain strains of *B. cereus type I, B. cereus type II, B. megaterium, B. sphaericus* and *B. mycoides* which were not isolated at a water temperature of 56°C (Tables 1-50). Due to the emergence of these *Bacillus spp.* at the elevated preparation temperature of 90°C, it may be possible that these organisms originated from slow germinating spores.

Pasteurising samples prior to storage did not significantly (at the P0.05 level) alter the mean microbiological quality over the course of the study. However, where the Total Aerobic Mesophilic Count (CFU g^{-1}) for a sample prepared at 90°C was significantly higher or lower compared to the same sample reconstituted at 56°C, then this additional heat treatment (i.e. 62.8°C for 30 mins) did influence the microbial profile by augmenting the difference between counts.

By far the most influential parameters effecting the type, number and concentration of *Bacillus* spp. were the duration and temperature of incubation. Storage temperatures at or below 10°C were unsuitable for growth of all 16 *Bacillus* spp. isolated in this study (P0.05). Examination of Table 5.10 revealed that the greater the storage temperature (above 20°C) and the longer the incubation period (≥8 hours), the larger the cell concentration (CFU g^{-1}) observed. Both Table 5.10 and Figure 5.4 show the effect of duration and temperature of storage on the concentration of cells achieved. This table reveals a consistently low mean Total Aerobic Mesophilic Count of approximately $\leq 5.0 \times 10^2$ CFU g^{-1} for IMF sampled immediately after reconstitution, which suggests that the various methods of preparation, cooling and storage did not significantly influence the microbiological quality of the reconstituted powder prior to subsequent storage.

Table 5.9 (a) Isolation of *Bacillus* spp. from 10 different reconstituted IMF products over a 24 hour period.

Bacillus species	Time (hr)	Number and type of IMF product supporting growth over 24 hrs										Total No. (n)[2]
		A	B	C	D	E	F	G	H	I	J	
B. cereus I	0	4	-	1	-	2	-	-	3	-	-	10
	8	5	4 (1*)	1	2	6	2	3	5 (1*)	1 (1*)	1	30
	14	5	4 (1*)	1	2	6	2	3	5 (1*)	2 (1*)	1	31
	24	5	4 (1*)	1	2	6	2	3	5 (1*)	2 (1*)	1	31
B. cereus II	0	1	1	-	-	1	-	1	2	-	1	7
	8	3	3	4 (3*)	4 (1*)	5	6 (1*)	4	4 (1*)	-	1	34
	14	3	5	4 (3*)	4 (1*)	5	6	4	4 (1*)	1	1	37
	24	3	5	4 (3*)	4 (1*)	5	6 (1*)	4	5 (1*)	1	1	38
B. licheniformis	0	6	8	6	6	5	6	4	3	1	1	46
	8	6	8	6	6	5	6	4	3	1	1	46
	14	6	8	6	6	5	6	5	3	1	1	47
	24	4	6	5	4	3	5	2	2	-	-	31
B. subtilis	0	4	3	-	3	4	5	5	5	1	-	30
	8	7	3	1	3	4	6	6	5	1	-	36
	14	7	3	1	3	4	6	6	5	1	-	36
	24	6	2	1	3	3	5	6	5	1	-	32
B. pumilus	0	-	1	2	2	-	1	2	-	-	1	9
	8	-	2	3	2	-	2	3	-	-	1	13
	14	-	2	3	2	-	2	3	-	-	1	13
	24	-	2	3	2	-	2	3	-	-	1	13
B. megaterium	0	1	-	1	-	1	-	-	-	-	-	3
	8	1	1	2	1	1	2	1	2 (1*)	1 (1*)	-	12
	14	1	1	2	1	2 (1*)	2	1	2 (1*)	1 (1*)	-	13
	24	1	1	2	1	2 (1*)	2	1	2 (1*)	1 (1*)	-	13

* Possibly slow germinating spores

: Total number of IMF analysed over the duration of the study came to 100

(A) SMA White Cap (B) SMA Gold Cap (C) Milupa Aptamil (D) Milupa Milumil (E) Nutrilon Premium (F) Nutrilon Plus (G) Farleys Oster Milk One (H) Farleys Oster Milk Two (I) Farleys First Milk (J) Farleys Second Milk

Table 5.9 (b) Isolation of *Bacillus* spp. from 10 different reconstituted IMF products over a 24 hour storage period.

Bacillus species	Time (hr)	Number and type of IMF product supporting growth over 24 hrs										Total No. (n)[‡]
		A	B	C	D	E	F	G	H	I	J	
B. sphaericus	0	1	1	1	3	1	-	-	1	-	-	8
	8	1	3	1	3	3	3	3	3	-	-	20
	14	1	3	1	3	3 (2*)	3	3	3 (2*)	1 (1*)	-	21
	24	1 (1*)	3	1	3	3 (2*)	3	3	3 (2*)	1 (1*)	-	21
B. mycoides	0	-	-	1	1	-	-	1	1	1	-	5
	8	2 (1*)	1	2	1	1	-	3 (1*)	1	1	-	12
	14	2 (1*)	2	2	1	1	-	3 (1*)	1	1	-	13
	24	2 (1*)	2	2	1	1	-	3 (1*)	1	1	-	13
B. amylioliquefaciens	0	1	-	-	-	-	-	-	-	-	-	1
	8	1	-	-	-	-	-	-	1	-	-	2
	14	1	-	-	-	-	-	-	1	-	-	2
	24	1	-	-	-	-	-	-	1	-	-	2
B. lentus	0	-	-	-	-	-	-	-	-	-	-	0
	8	-	-	1	-	1	-	1	-	-	-	3
	14	-	-	1	-	1	-	1	-	-	-	3
	24	-	-	1	-	1	-	1	-	-	-	3
B. coagulans	0	-	-	1	-	1	1	-	3	-	-	3
	8	-	-	1	-	1	2	-	3	-	1	8
	14	-	-	1	-	1	2	-	3	-	1	8
	24	-	-	1	-	1	2	-	3	-	1	8
B. circulans 2	0	1	2	-	-	-	-	-	1	-	-	4
	8	1	2	-	-	-	1	-	1	1	-	6
	14	1	2	-	-	-	1	-	1	1	-	6
	24	1	2	-	-	-	1	-	1	1	-	6

* Possibly slow germinating spores

‡ Total number of IMF analysed over the duration of the study came to 100

(A) SMA White Cap (B) SMA Gold Cap (C) Milupa Aptamil (D) Milupa Milumil (E) Nutrilon Premium (F) Nutrilon Plus (G) Farleys Oster Milk One (H) Farleys Oster Milk Two (I) Farleys First Milk (J) Farleys Second Milk

Table 5.9 (c) Isolation of *Bacillus* spp. from 10 different reconstituted IMF products over a 24 hour storage period.

Bacillus species	Time (hr)	Number and type of IMF product supporting growth over 24 hrs										Total No. (n)[‡]
		A	B	C	D	E	F	G	H	I	J	
B. brevis	0	-	-	-	1	-	-	-	-	-	-	1
	8	-	-	-	1	-	-	2	-	-	-	3
	14	-	-	-	1	-	-	2	-	-	-	3
	24	-	-	-	1	-	-	2	-	-	-	3
B. laterosporus	0	-	-	-	-	-	-	-	-	-	-	0
	8	-	-	-	-	-	-	-	-	-	-	0
	14	-	-	1	-	-	-	-	-	-	-	1
	24	-	-	1	-	-	-	-	-	-	-	1
B. polymyxa	0	-	-	2	1	-	-	-	-	-	-	3
	8	-	-	2	2	-	-	-	-	-	-	4
	14	-	-	2	2	-	-	-	-	-	-	4
	24	-	-	2	2	-	-	-	-	-	-	4
B. firmus	0	1	-	-	-	-	1	-	2	-	-	4
	8	1	-	-	-	-	1	-	2	-	-	4
	14	1	-	-	-	-	1	-	2	-	-	4
	24	1	-	-	-	-	1	-	2	-	-	4
No Bacillus spp detected	0	1	1	-	-	1	-	2	-	1	-	6
	8	-	1	-	-	-	-	-	-	1	1	2
	14	-	1	-	-	-	-	-	-	-	-	1
	24	-	1	-	-	-	-	-	-	-	-	1
Other organisms (Cocci)	0	-	-	1	-	-	-	1	-	-	1	3
	8	-	-	1	-	-	2	2	-	-	1	6
	14	-	-	1	-	-	2	2	-	-	1	6
	24	-	-	1	-	-	2	2	-	-	1	6

* Possibly slow germinating spores

‡ Total number of IMF analysed over the duration of the study came to 100

(A) SMA White Cap (B) SMA Gold Cap (C) Milupa Aptamil (D) Milupa Milumil (E) Nutrilon Premium (F) Nutrilon Plus (G) Farleys Oster Milk One (H) Farleys Oster Milk Two (I) Farleys First Milk (J) Farleys Second Milk

Table 5.10 illustrates the mean Total Aerobic Mesophilic Count (CFU g^{-1} IMF powder) for all the IMF samples analysed at a particular storage temperature and time and under a defined set of preparation and cooling conditions.

Storage Temp (°C)	Recon† Temp (°C)	Mean Total Aerobic Mesophilic Count (CFU ml^{-1})‡											
		Tap Cooled				Table Top Cooled				Refrigerated			
		24 h	14 h	8 h	0 h	24 h	14 h	8 h	0 h	24 h	14 h	8 h	0 h
35	56	**$1.08x10^8$**	**$1.75x10^4$**	**$2.5x10^3$**	**$2.5x10^2$**	**$1.98x10^8$**	**$2.32x10^4$**	**$2.82x10^3$**	**$2.85x10^2$**	**$1.72x10^8$**	**$2.46x10^3$**	**$2.61x10^3$**	**$2.0x10^2$**
	(Range)	($9.0x10^5$-$8.5x10^8$)	($5.0x10^2$-$8.5x10^4$)	($1.0x10^2$-$1.9x10^4$)	(NDx10^2-$1.7x10^3$)	($2.5x10^6$-$1.9x10^8$)	($8.0x10^2$-$9.43x10^4$)	(NDx10^2-$1.7x10^4$)	(NDx10^2-$106x10^3$)	($2.8x10^6$-$2.9x10^8$)	($3.0x10^2$-$3.1x104$)	(NDx10^2-$5.3x10^3$)	(NDx10^2-$5.0x10^2$)
	90	**$1.15x10^8$**	**$1.81x10^4$**	**$2.72x10^3$**	**$2.8x10^2$**	**$9.67x10^7$**	**$1.87x10^4$**	**$2.57x10^3$**	**$3.2x10^2$**	**$9.11x10^7$**	**$2.6x10^3$**	**$2.7x10^3$**	**$2.11x10^2$**
	(Range)	($3.5x10^6$-$7.0x10^8$)	($5.0x10^2$-$8.5x10^4$)	($5.0x10^2$-$1.7x10^4$)	(NDx10^2-$2.1x10^3$)	($1.9x10^5$-$7.5x10^8$)	($6.0x10^2$-$8.1x10^4$)	($1.0x10^2$-$1.6x10^4$)	(NDx10^2-$1.9x10^3$)	($1.2x10^7$-$4.9x10^8$)	($5.0x10^2$-$1.1x10^4$)	(NDx10^2-$6.1x10^3$)	(NDx10^2-$5.0x10^2$)
30	56	**$8.4x10^6$**	**$4.83x10^3$**	**$1.31x10^3$**	**$1.81x10^2$**	**$8.7x10^6$**	**$5.15x10^3$**	**$1.7x10^3$**	**$5.5x10^2$**	**$1.8x10^7$**	**$5.0x10^3$**	**$8.3x10^2$**	**$1.0x10^2$**
	(Range)	($1.0x10^5$-$1.2x10^7$)	($6x10^2$-$1.19x10^4$)	($2.0x10^2$-$5.0x10^3$)	(NDx10^2-$3.0x10^3$)	($7.8x10^5$-$3.0x10^7$)	($5.0x10^2$-$1.4x10^4$)	($1.0x10^2$-$6.1x10^3$)	(NDx10^2-$2.1x10^3$)	($3.0x10^5$-$5.1x10^7$)	($2.3x10^3$-$9.3x10^3$)	($4.0x10^2$-$1.4x10^3$)	(NDx10^2-$2.0x10^2$)
	90	**$1.07x10^7$**	**$5.59x10^3$**	**$1.61x10^3$**	**$2.05x10^2$**	**$1.21x10^7$**	**$4.86x10^3$**	**$7.1x10^2$**	**$2.13x10^2$**	**$2.6x10^7$**	**$4.4x10^3$**	**$8.3x10^2$**	**$1.0x10^2$**
	(Range)	($5.0x10^5$-$4.5x10^7$)	($3.0x10^2$-$2.4x10^4$)	($3.0x10^2$-$6.7x10^3$)	(NDx10^2-$1.2x10^3$)	($8.1x10^5$-$6.83x10^7$)	($5.0x10^2$-$1.33x10^4$)	($1.0x10^2$-$7.1x10^3$)	(NDx10^2-$1.6x10^3$)	($2.1x10^5$-$7.0x10^7$)	($9.0x10^2$-$8.7x103$)	(3.010^2-$3.0x10^3$)	(NDx10^2-$3.0x10^2$)
25	56	**$2.49x10^6$**	**$3.82x10^3$**	**$5.98x10^2$**	**$4.33x10^2$**	**$5.8x10^5$**	**$1.21x10^3$**	**$4.5x10^2$**	**$2.2x10^3$**	**$7.5x10^5$**	**$9.3x10^2$**	**$2.75x10^2$**	**$1.25x10^2$**
	(Range)	($2.9x10^3$-$5.8x10^7$)	($1.0x10^2$-$3.52x10^4$)	(NDx10^2-$7.43x10^2$)	(NDx10^2-$6.1x10^2$)	($3.0x10^4$-$1.6x10^6$)	($2.0x10^2$-$3.6x10^3$)	(NDx10^2-$2.3x10^3$)	(NDx10^2-$1.8x10^3$)	($6.5x10^4$-$2.7x10^6$)	($8.0x10^2$-$1.6x103$)	($1.0x10^2$-$5.0x10^2$)	(NDx10^2-$3.0x10^2$)
	90	**$1.23x10^6$**	**$3.06x10^3$**	**$6.46x10^2$**	**$5.2x10^2$**	**$6.67x10^5$**	**$1.92x10^3$**	**$9.5x10^2$**	**$2.4x10^2$**	**$5.4x10^5$**	**$1.1x10^3$**	**$3.6x10^2$**	**$1.36x10^2$**
	(Range)	($4.0x10^2$-$1.36x10^7$)	(NDx10^2-$4.12x10^4$)	(NDx10^2-$4.6x10^3$)	(NDx10^2-$5.6x10^3$)	($8.2x10^4$-$1.6x10^6$)	($3.0x10^2$-$3.3x10^4$)	(NDx10^2-$3.0x10^3$)	(NDx10^2-$1.6x10^3$)	($5.9x10^4$-$1.9x10^6$)	($6.0x10^2$-$1.8x10^3$)	($3.0x10^2$-$5.0x10^2$)	(NDx10^3-$4.0x10^2$)
20	56	**$1.72x10^3$**	**$5.4x10^2$**	**$2.28x10^2$**	**$2.0x10^2$**	**$1.3x10^3$**	**$5.8x10^2$**	**$2.38x10^2$**	**$2.11x10^2$**	**$1.52x10^3$**	**$5.6x10^2$**	**$2.13x10^2$**	**$2.1x10^2$**
	(Range)	($4.0x10^2$-$4.4x10^3$)	($1.0x10^2$-$2.1x10^3$)	(NDx10^2-$1.5x10^3$)	(NDx10^2-$1.5x10^3$)	($5.0x10^2$-$3.2x10^3$)	($1.0x10^2$-$2.3x10^2$)	(NDx10^2-$2.5x10^3$)	(NDx10^2-$1.9x10^3$)	($5.0x10^2$-$3.9x10^3$)	($1.0x10^2$-$1.5x10^3$)	(NDx10^2-$1.4x10^3$)	(NDx10^2-$1.6x10^2$)
	90	**$1.82x10^3$**	**$4.63x10^2$**	**$2.15x10^2$**	**$1.95x10^2$**	**$9.1x10^2$**	**$5.2x10^2$**	**$2.41x10^2$**	**$2.06x10^2$**	**$1.67x10^3$**	**$5.0x10^2$**	**$2.23x10^2$**	**$1.9x10^2$**
	(Range)	($6.0x10^2$-$4.2x10^3$)	(NDx10^2-$2.2x10^3$)	(NDx10^2-$1.3x10^3$)	(NDx10^2-$1.7x103$)	(NDx10^2-$2.4x10^3$)	(NDx10^2-$1.8x10^3$)	(NDx10^2-$1.8x10^3$)	(NDx10^2-$1.7x10^3$)	($1.0x10^2$-$2.8x10^3$)	(NDx10^2-$2.3x10^3$)	(NDx10^2-$1.5x10^3$)	(NDx10^2-$1.3x10^2$)
≤10	56	**$2.4x10^2$**	**$2.23x10^2$**	**$1.8x10^2$**	**$2.21x10^2$**	**$3.2x10^2$**	**$2.11x10^2$**	**$2.53x10^2$**	**$1.95x10^2$**	**$2.46x10^2$**	**$2.6x10^2$**	**$1.8x102$**	**$2.0x10^2$**
	(Range)	(NDx10^2-$3.5x10^3$)	(NDx10^2-$4.0x10^3$)	(NDx10^2-$3.5x10^3$)	(NDx10^2-$2.9x10^3$)	(NDx10^2-$2.6x10^2$)	(NDx10^2-$2.1x10^2$)	(NDx10^2-$2.3x10^3$)	(NDx10^2-$3.0x10^2$)	(NDx10^2-$2.5x10^2$)	(NDx10^2-$1.8x10^3$)	(NDx102-$1.7x10^2$)	(NDx10^2-$1.5x10^3$)
	90	**$1.86x10^2$**	**$2.55x10^2$**	**$2.23x10^2$**	**$1.9x10^2$**	**$2.01x10^2$**	**$2.51x10^2$**	**$2.68x10^2$**	**$2.16x10^2$**	**$2.2x10^2$**	**$2.41x10^2$**	**$1.95x10^2$**	**$2.15x10^2$**
	(Range)	(NDx10^2-$3.0x10^2$)	(NDx10^2-$3.1x10^2$)	(NDx10^2-$2.5x10^3$)	(NDx10^2-$2.8x10^3$)	(NDx10^2-$2.1x10^3$)	(NDX10^2-$1.8x10^3$)	(NDx10^2-$2.1x10^3$)	(NDx10^2-$2.5x10^2$)	(NDx10^2-$2.6x10^3$)	(NDx10^2-$1.7x10^3$)	(NDx10^2-$1.5x10^3$)	(NDx10^2-$2.0x10^3$)

† Mean total aerobic counts (CFU ml^{-1}) are highlighted in bold, while the variation about the mean is represented by the range (illustrated below the mean).

The "range" illustrated in Table 5.10 refers to the lowest and highest recorded count (CFU g^{-1}) achieved at that particular combination of temperature and time for an individual IMF sample. The results from Tables 1-50 showed that irrespective of the preparation, cooling and storage conditions, the Total Aerobic Count at each sample time period largely depended on the type, number and concentration of organism present at the early stages of storage. Some samples contained only one *Bacillus* spp. at either a low ($<10^2$ CFU g^{-1}) or moderate ($> 10^2$ CFU g^{-1}) cell concentration, while others consisted of up to 5 different *Bacillus* spp.

However, these tables further revealed that the type of *Bacillus* spp. present in samples after 24 hours at storage temperatures ≥25°C often significantly varied from the initial indigenous flora at 0 hours (P0.05). While overnight incubation of samples at or below 20°C did not significantly alter the type of *Bacillus* spp. present. This variation in *Bacillus* spp. was seen to be related to the concentration and type of organisms detected in the early stages of storage (≥8 hours), especially where *B. cereus* was recovered.

Although *B. cereus type I* and *II* were recovered in a low number of IMF samples after the initial formula preparation (Table 5.7), these two potential food borne pathogens often grew to the exclusion of other *Bacillus spp.* when held at ≥25°C for 24 hours. Moreover, incubation of samples which were containing both *B. cereus* and *B. licheniformis* for ≥14 hours at temperatures greater than 25°C resulted in the inhibition of the latter organism in all IMF samples analysed. Where antagonism appeared to be evident in these co-habitated samples, *B. licheniformis* was kept to a cell concentration of approximately 10^4 CFU g^{-1}. Cultivation of both organisms in the same TSYEB and/or tyndallised IMF under controlled conditions confirmed that *B. cereus* exhibits an inhibitory effect on *B. licheniformis*, where Figures 5.5 (a), 5.5 (b) and 5.5 (c) reveal the inhibition of *B. licheniformis* after 14 hours at 25°C, 30°C and 35°C respectively. In these figures, *B. licheniformis* was cultivated in duplicate with (total count) and without (Bl-Bc) *B. cereus*, and the concentration of *B. licheniformis* in the shared IMF sample (Bl + Bc) is also shown. Two other members of the *B. subtilis* group (i.e. *B. subtilis* and *B. pumilus*) also demonstrated a reduction in cell concentration (CFU g^{-1}) at 24 hours at temperatures ≥25°C when grown in the

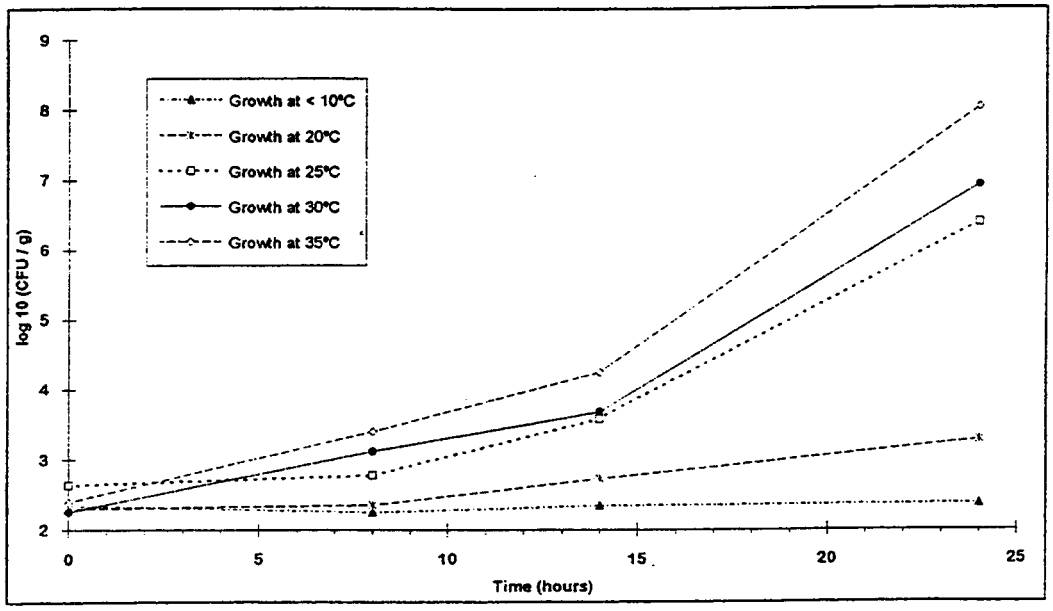


Figure 5.4 illustrates the mean Total Aerobic Mesophilic Count (CFU g^{-1}) for the reconstituted IMF samples stored at various temperatures over a 24 hour period.

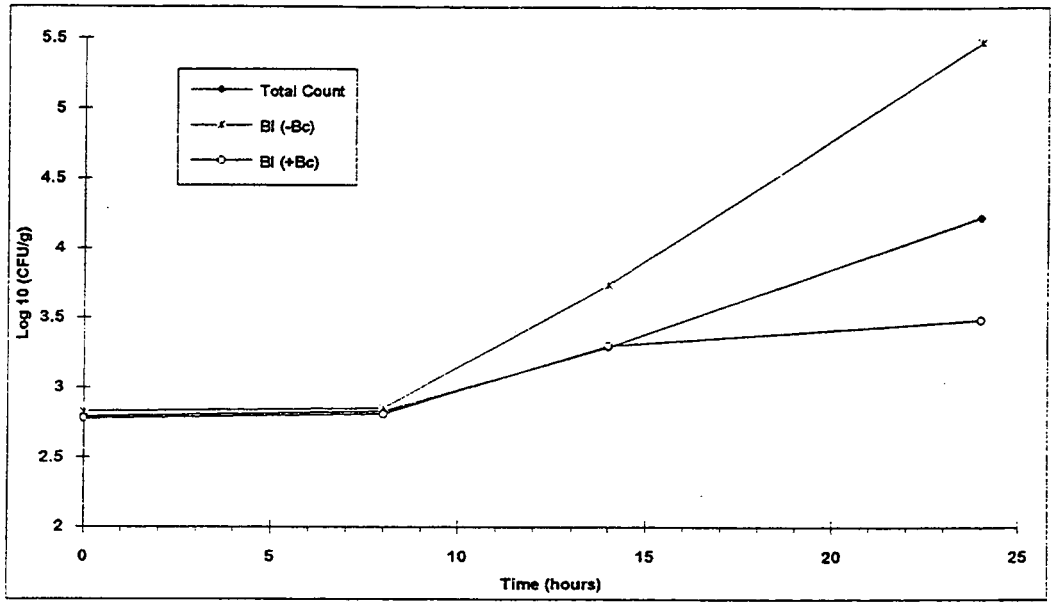


Figure 5.5 (a) Growth profile of *B. licheniformis* sharing (designated as Bl+Bc) and not sharing (designated as Bl-Bc) the same IMF environment with *B. cereus* over a 24 hour period at 25°C.

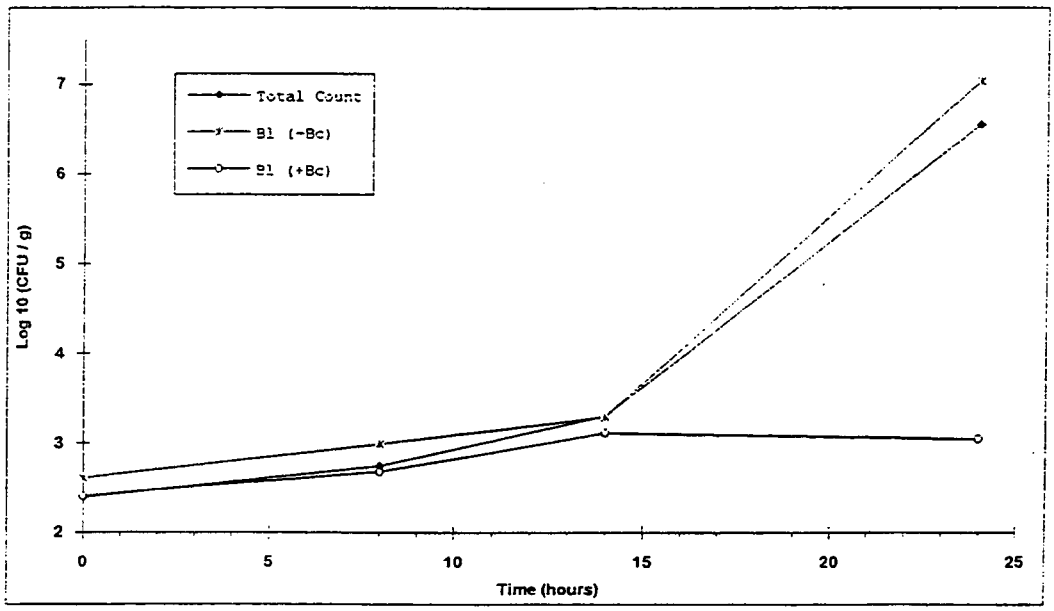


Figure 5.5 (b) Growth profile of *B. licheniformis* sharing (designated as Bl+Bc) and not sharing (designated as Bl-Bc) the same IMF environment with *B. cereus* over a 24 hour period at 30°C.

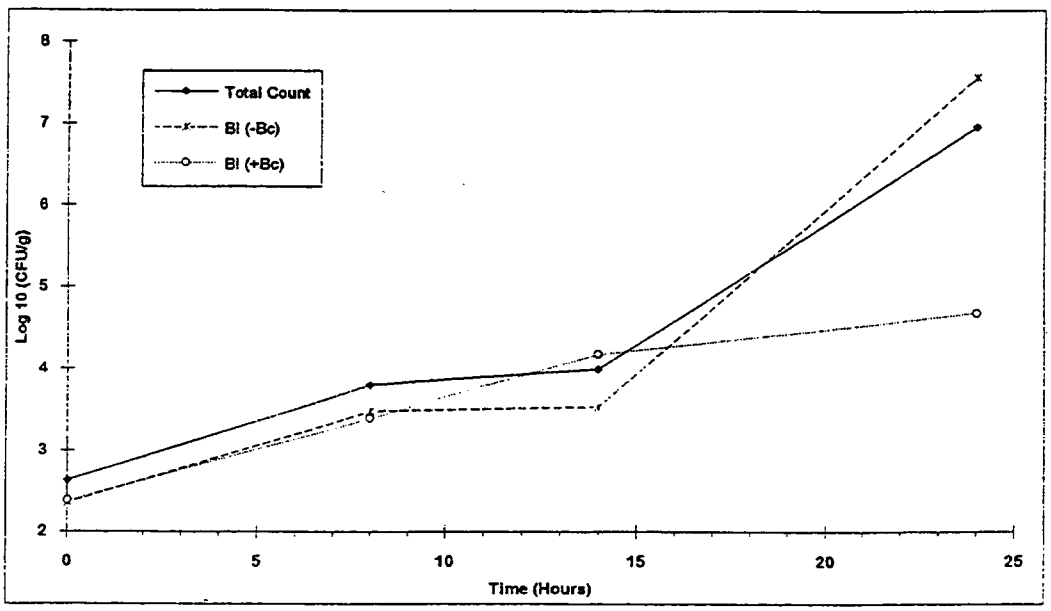


Figure 5.5 (c) Growth profile of *B. licheniformis* sharing (designated as Bl+Bc) and not sharing (designated as Bl-Bc) the same IMF environment with *B. cereus* over a 24 hour period at 35°C.

presence of *B. cereus*. Possible antagonism towards these organisms due to interactions with *B. cereus* and/or other *Bacillus spp.* was not examined.

The mean Total Aerobic Mesophilic Count for *B. cereus I* and *II* in samples prepared at either 56°C or 90°C, cultivated at a variety of storage temperatures over a 24 hour period is shown in Tables 5.11 and 5.12 respectively. These tables show the *B. cereus* cell concentration in samples where *B. licheniformis* was either present or absent and may serve as a predictive tool as to the possible levels of microbial growth (CFU g^{-1}) reached in infant formulae under various preparation and storage conditions. While the Total Aerobic Mesophilic Count for *B. cereus I* and *II* was not influenced by the presence of *B. licheniformis*, Table 5.13 reveals that the concentration of *B. licheniformis* recovered after 24 hours growth at temperatures >25°C was significantly lower (P 0.05) where this organism shared the same environment with *B. cereus I* and/or *II*.

Tables 5.14 (a) and (b) show the total number of samples to be colonised by different concentrations (CFU g^{-1}) of *B. cereus I* and *II* over a 24 hour period at both 25°C and 35°C. The incubation of samples at 35°C resulted in a greater percentage of infant formulae containing higher levels of these aerobic spore formers. Furthermore, storage of samples containing *B. cereus* for ≥ 14 hours at 25°C resulted in some infant formulae surpassing the recommended Total Aerobic Mesophilic Count of ≥ $1.0x10^4$ CFU g^{-1} (ICMSF 1986).

Diarrhoegenic toxin production was exhibited by 10 of the 38 *B. cereus* type II strains (isolated from the reconstituted infant formulae) cultivated in BHI broth under orbital conditions (110 rpm) after 14 and 24 hours (section 3.9). Moreover, of these 38 *B. cereus II* strains, 5 and 1 (enterotoxin producers) and 12 and 3 (non-enterotoxin producers) exhibited growth on TSYEA and *Bacillus cereus* Selective Agar (BCSA) at 8°C and 5°C respectively over a 10 day incubation period (Table 5.15). Due to the fat composition of the IMF samples interfering with the RPLA enterotoxin assay, toxin production by these *B. cereus II* strains could not be demonstrated in IMF at this point in time.

Table 5.11 Total Aerobic Mesophilic Count (CFU g^{-1} powder) for *B. cereus I* (with and without *B. licheniformis* in the same reconstituted IMF sample) over a 24 hour period at a variety of storage temperatures.

Storage Temp (°C)	Population Effect	Total Aerobic Count (cfu g^{-1}) for *B. cereus I* over a 24 hrs post -reconstitution at 56 and 90°C							
		24 hr		14 hr		8 hr		0 hr	
		56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C
35	Total Count¶	**$6.36x10^7$**	**$5.43x10^7$**	**$7.81x10^3$**	**$1.17x10^4$**	**$6.4x10^2$**	**$7.0x10^2$**	**$2.0x10^1$**	**$4.6x10^1$**
	(Range)	ND (10^2)-$2.0x10^8$	$5.2x10^5$-$2.1x10^8$	ND (10^2)-$5.1x10^4$	$2.0x10^2$-$3.9x10^4$	ND (10^2)-$1.9x10^3$	ND (10^2)-$2.6x10^3$	ND (10^2)-$1.0x10^2$	ND (10^2)-$3.0x10^2$
	Bc (-Bl)§	**$9.47x10^7$**	**$1.1x10^8$**	**$1.7x10^4$**	**$1.9x10^4$**	**$7.7x10^2$**	**$1.2x10^3$**	**$2.5x10^1$**	**$5.0x10^1$**
	(Range)	ND (10^2)-$2.0x10^8$	$6.6x10^7$-$2.1x10^8$	$8.0x10^2$-$5.1x10^4$	$1.1x10^4$-$3.9x10^4$	$1.0x10^2$-$1.9x10^3$	$2.0x10^2$-$2.6x10^3$	ND (10^2)-$1.0x10^2$	ND (10^2)-$2.0x10^2$
	Bc (+Bl)¥	**$4.11x10^7$**	**$8.0x10^7$**	**$2.2x10^3$**	**$6.45x10^3$**	**$4.5x10^2$**	**$3.0x10^2$**	**$2.5x10^1$**	**ND (10^2)**
	(Range)	$2.46x10^6$-$6.1x10^7$	$5.2x10^5$-$2.1x10^8$	ND 10^2) $3.9x10^4$	$2.0x10^2$-$2.5x10^4$	ND (10^2)-$6.0x10^2$	ND (10^2)-$6.0x10^2$	ND (10^2)-$1.0x10^2$	ND (10^2)
30	Total Count	**$2.14x10^7$**	**$1.49x10^7$**	**$1.52x10^3$**	**$1.26x10^3$**	**$3.5x10^2$**	**$1.4x10^2$**	**ND (10^2)**	**ND (10^2)**
	(Range)	$6.0x10^6$-$5.3x10^7$	$5.1x10^5$-$4.54x10^7$	$6.0x10^2$-$1.9x10^4$	$4.0x10^2$-$2.1x10^3$	ND 10^2)-$1.3x10^3$	ND (10^2)-$2.0x10^2$	ND (10^2)	ND (10^2)
	Bc (-Bl)	**$2.32x10^7$**	**$1.49x10^7$**	**$1.3x10^3$**	**$1.47x10^3$**	**$2.8x10^2$**	**$7.7x10^7$**	**ND (10^2)**	**ND (10^2)**
	(Range)	$6.0x10^2$-$5.3x10^7$	$9.5x10^5$-$4.5x10^7$	$6.0x10^2$-$1.9x10^3$	$4.0x10^2$-$2.1x10^3$	ND (10^2)-$1.3x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)	ND (10^2)
	Bc(+Bl)	**$1.06x10^7$**	**$8.6x10^6$**	**$1.0x10^3$**	**$1.1x10^3$**	**$1.0x10^2$**	**$2.0x10^2$**	**ND (10^2)**	**ND (10^2)**
	(Range)	$7.0x10^2$-$3.3x10^7$	$5.1x10^5$-$2.2x10^7$	$8.0x10^2$-$1.6x10^3$	$4.0x10^2$-$2.0x10^3$	ND (10^2)-$1.1x10^3$	ND (10^2)-$5.0x10^2$	ND (10^2)	ND (10^2)
25	Total Count	**$4.1x10^6$**	**$8.4x10^5$**	**$3.1x10^2$**	**$3.52x10^2$**	**$5.7x10^1$**	**$5.8x10^1$**	**$5.2x10^1$**	**$1.78x10^1$**
	(Range)	ND (10^2)-$5.6x10^7$	$8.0x10^2$-$1.1x10^7$	ND (10^2)-$1.2x10^3$	ND (10^2)-$8.0x10^2$	ND (10^2)-$3.0x10^2$	ND (10^2)-$3.0x10^2$	ND (10^2)-$4.0x10^2$	ND (10^2)-$1.0x10^2$
	Bc(-Bl)	**$1.9x10^6$**	**$1.2x10^6$**	**$2.7x10^2$**	**$3.2x10^2$**	**$3.3x10_1$**	**$4.5x10^1$**	**$4.2x10^1$**	**8.0**
	(Range)	ND (10^2)-$1.3x10^7$	ND (102)-$1.1x10^7$	ND (10^2)-$1.2x10^3$	ND (10^2)-$8.0x10^2$	ND (10^2)-$3.0x10^2$	ND (10^2)-$3.0x10^2$	ND (10^2)-$4.0x10^2$	ND (10^2)-$1.0x10^2$
	Bc(+Bl)	**$8.1x10^6$**	**$1.1x10^6$**	**$3.66x10^2$**	**$3.26x10^2$**	**$6.25x10^1$**	**$7.5x10^1$**	**$4.2x10^1$**	**$2.5x10^1$**
	(Range)	$5.1x10^4$-$5.6x10^7$	1.0x103-$1.3x10^7$	$1.0x10^2$-$8.0x10^2$	$1.0x10^2$-$1.0x10^3$	ND (10^2)-$2.0x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)-$1.0x10^2$
20	Total Count	**$5.6x10^2$**	**$6.0x10^2$**	**$8.3x10^1$**	**$6.6x10^1$**	**$2.0x10^1$**	**ND (10^2)**	**$1.6x10^1$**	**$1.6x10^1$**
≤ 10	Total Count	**$2.0x10^1$**	**$3.3x10^1$**	**$2.0x10^1$**	**$1.6x10^1$**	**$4.6x10^1$**	**ND (10^2)**	**$2.0x10^1$**	**$2.5x10^1$**

¶ Mean Total count for *B. cereus I*§ Mean Total count for *B. cereus I* when not competing with *B. licheniformis*

¥ Mean Total count for *B. cereus I* when in direct competition with *B. licheniformis*

Table 5.12 Total Aerobic Mesophilic Count (CFU g^{-1} powder) for *B. cereus II* (with and without *B. licheniformis* in the same reconstituted IMF sample) over a 24 hour period at a variety of storage temperatures.

Storage Temp (°C)	Population Effect	Total Aerobic Count (cfu g^{-1}) for *B. cerues II* over a 24 hrs post -reconstitution at 56 and 90°C							
		24 hr		14 hr		8 hr		0 hr	
		56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C
35	Total Count¶	**$1.28x10^8$**	**$1.45x10^8$**	**$9.91x10^3$**	**$1.1x10^4$**	**$1.77x10^3$**	**$1.55x10^3$**	**$2.3x10^1$**	**$1.4x10^2$**
	(Range)	ND (10^2)-$7.9x10^8$	$4.0x10^5$-$5.0x10^8$	ND (10^2)-$6.1x10^4$	ND (10^2)-$4.5x10^4$	ND (10^2)-$8.0x10^2$	$1.0x10^2$-$6.9x10^3$	ND (10^2)-$2.0x10^2$	ND (10^2)-$4.0x10^2$
	Bc (-Bl)§	**$4.1x10^7$**	**$1.7x10^8$**	**$1.4x10^4$**	**$1.7x10^4$**	**$1.66x10^3$**	**$1.56x10^3$**	**$3.75x10^1$**	**$1.5x10^2$**
	(Range)	$2.1x10^7$-$7.9x10^8$	$3.5x10^6$-$5.0x10^8$	ND (10^2)-$6.1x10^4$	$8.0x10^2$-$4.1x10^4$	ND (10^2)-$8.0x10^3$	$2.0x10^2$-$4.1x10^4$	ND (10^2)-$2.0x10^2$	ND (10^2)-$4.0x10^2$
	Bc (+Bl)¥	**$5.52x10^7$**	**$8.05x10^7$**	**$2.0x10^3$**	**$4.2x10^3$**	**$1.5x10^2$**	**$2.0x10^2$**	**ND (10^2)**	**ND (10^2)**
	(Range)	$2.0x10^7$-$1.3x10^8$	$2.1x10^7$-$5.0x10^8$	$2.0x10^2$-$9.3x10^3$	ND (10^2)-$1.0x10^4$	NDF (10^2)-$9.0x10^2$	ND (10^2)-$1.1x10^3$	ND (10^2)	ND (10^2)
30	Total Count	**$5.9x10^6$**	**$7.3x10^6$**	**$1.68x10^4$**	**$9.6x10^3$**	**$1.48x10^3$**	**$1.35x10^3$**	**$6.2x10^1$**	**$2.0x10^1$**
	(Range)	ND (10^2)-$1.8x10^7$	$4.0x10^5$-$2.1x10^7$	ND (10^2)-$6.4x10^4$	$3.0x10^2$-$4.5x10^4$	ND (10^2)-$8.0x10^4$	$1.0x10^2$-$6.9x10^3$	ND (10^2)-$2.0x10^2$	ND (10^2)-$2.0x10^2$
	Bc (-Bl)	**$5.5x10^6$**	**$3.1x10^6$**	**$3.0x10^3$**	**$9.0x10^2$**	**$2.0x10^2$**	**$1.5x10^2$**	**ND (10^2)**	**ND (10^2)**
	(Range)	ND (10^2)-$2.1x10^7$	$1.0x10^5$-$1.6x10^7$	$3.0x10^2$-$5.4x10^3$	ND (10^2)-$2.3x10^3$	ND (10^2)-$3.0x10^2$	$1.0x10^2$-$2.0 x10^2$	ND (10^2)	ND (10^2)
	Bc(+Bl)	**$6.8x10^6$**	**$7.3x10^5$**	**$1.36x10^3$**	**$5.3x10^2$**	**$1.5x10^2$**	**$1.5x10^2$**	**ND (10^2)**	**ND (10^2)**
	(Range)	$1.0x10^5$-$1.8x10^7$	$4.0x10^5$-$1.0x10^6$	$7.0x10^2$-$2.4x10^3$	$3.0x10^2$-$8.0 x10^2$	$1.0x10^2$-$2.0x10^2$	$1.0x10^2$-$2.0x10^2$	ND (10^2)	ND (10^2)
25	Total Count	**$1.08x10^6$**	**$6.8x10^5$**	**$3.8x10^2$**	**$3.5x10^2$**	**$4.1x10^1$**	**$6.3x10^1$**	**$2.0x10^1$**	**5.0x10**
	(Range)	$4.1x10^4$-$1.5x10^7$	$2.0x10^4$-$8.5x10^6$	ND (10^2)-$8.0x10^2$	ND (10^2)-$1.0x10^3$	ND (10^2)-$2.0x10^2$	ND (10^2)-$5.0x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)-$1.0x10^2$
	Bc(-Bl)	**$1.6x10^6$**	**$1.3x10^6$**	**$4.0x10^2$**	**$3.75x10^2$**	**$6.42x10^1$**	**$5.8x10^1$**	**$1.2x10^1$**	**ND (10^2)**
	(Range)	2.0x10-$1.5x10^{-4}$	$2.0x10^3$-$8.5x10^6$	ND (10^2)-$8.0x10^2$	ND (10^2)-$1.0x10^3$	ND (10^2)-$3.0x10^2$	ND (10^2)-$4.0x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)
	Bc(+Bl)	**$3.28x10^5$**	**$3.0x10^5$**	**$3.2x10^2$**	**$4.1x10^2$**	**$1.0x10^2$**	**$8.7x10^1$**	**$1.4x10^1$**	**$1.2x10^1$**
	(Range)	ND (10^2)-$2.1x10^6$	$4.1x10^4$-$2.1x10^6$	$2.0x10^2$-$6.0x10^2$	ND (10^2)-$8.0x10^2$	ND (10^2)-$2.0x10^2$	ND (10^2)-$4.0x10^2$	ND (10^2)-$1.0x10^2$	ND (10^2)-$1.0x10^2$
20	Total Count	**1.1x103**	**$5.0x10^2$**	**$5.0x10^1$**	**$6.6x10^1$**	**ND (10^2)**	**ND (10^2)**	**ND (10^2)**	**ND (10^2)**
≤ 10	Total Count	**ND (10^2)**	**$1.4x10^1$**	**ND (10^2)**	**$1.2x10^1$**	**ND (10^2)**	**$1.4x10^1$**	**5.0x10**	**$1.4x10^1$**

¶ Mean Total count for *B. cereus II*§ Mean Total count for *B. cereus II* when not competing with *B. licheniformis*

¥ Mean Total count for *B. cereus II* when in direct competition with *B. licheniformis*

Table 5.13 Total Aerobic Mesophilic Count (CFU g^{-1} powder) for *B. licheniformis* (with and without *B. cereus I* and/or *II* in the same reconstituted IMF sample) over a 24 hour period at a variety of storage temperatures.

Storage Temp (°C)	Population Effect	Total Aerobic Count (cfu g^{-1}) for *B. licheniformis* over a 24 hrs post reconstitution at 56 and 90°C							
		24 hr		14 hr		8 hr		0 hr	
		56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C
35	Total Count¶	**$9.5x10^6$**	**$2.6x10^6$**	**$1.0x10^4$**	**$2.95x10^3$**	**$6.3x10^3$**	**$5.65x10^3$**	**$4.3x10^2$**	**$9.3x10^2$**
	(Range)	ND (10^2) - $6.0x10^7$	ND (10^2)- $2.1x10^7$	ND (10^2)- $1.0x10^5$	ND (10^2)- $7.0x10^4$	$8.0x10^2$- $1.1x10^4$	$1.0x10^2$ $6.4x10^3$	ND (10^2)- $1.1x10^3$	ND (10^2)- $1.9x10^3$
	Bl (-Bc)§	**$3.8x10^7$**	**$2.1x10^7$**	**$3.52x10^3$**	**$1.56x10^3$**	**$3.05x10^3$**	**$6.0x10^2$**	**$2.25x10^2$**	**$1.6x10^2$**
	(Range)	$3.1x10^7$- $6.0x10^7$	$1.0x10^7$- $7.0x10^7$	$7.0x10^2$- $1.0x10^5$	$7.0x10^2$- $3.4x10^4$	$5.0x10^2$- $1.0x10^4$	$5.0x10^2$- $7.0x10^2$	$2.0x10^2$- $1.4x10^3$	ND (10^2)- $5.0x10^2$
	Bl (+Bc)¥	**$5.0x10^4$**	**$3.7x10^4$**	**$1.48x10^4$**	**$4.4x10^3$**	**$2.45x10^3$**	**$2.5x10^3$**	**$2.4x10^2$**	**$8.0x10^2$**
	(Range)	ND (102)- 1.0x105	$2.0x10^4$- $7.0x10^4$	ND (10^2)- $7.0x10^4$	$3.0x10^2$- $1.2x10^4$	ND (10^2)- $9.0x10^3$	$8.0x10^2$- $2.2x10^4$	ND (10^2)- $1.1x10^3$	ND (10^2)- $1.1x10^3$
30	Total Count	**$3.6x10^6$**	**$1.57x10^6$**	**$2.0x10^3$**	**$1.45x10^3$**	**$5.6x10^2$**	**$5.5x10^2$**	**$2.5x10^2$**	**$2.3x10^2$**
	(Range)	ND (10^2)- $3.0x10^7$	ND (10^2)- $8.0x10^6$	ND (10^2)- $3.4x10^3$	ND (10^2)- $2.3x10^4$	ND (10^2)- $2.8x10^3$	ND (10^2)- $2.6x10^3$	ND (10^2)- $1.3x10^3$	ND (10^2)- $1.8x10^3$
	Bl (-Bc)	**$1.09x10^7$**	**$4.3x10^6$**	**$2.18x10^3$**	**$1.95x10^3$**	**$9.8x10^2$**	**$1.0x10^3$**	**$4.1x10^2$**	**$4.6x10^2$**
	(Range)	$2.4x10^6$- $3.0x10^7$	$1.9x10^6$- $8.0x10^6$	$8.0x10^2$- $2.8x10^3$	$3.0x10^2$- $4.4x10^3$	$4.0x10^2$- $2.8x10^3$	$1.0x10^2$- $2.6x10^3$	ND (10^2)- $8.0x10^2$	ND (10^2)- $1.1x10^3$
	Bl (+Bc)	**$1.1x10^3$**	**ND (10^3)**	**$1.3x10^3$**	**$9.8x10^2$**	**$4.8x10^2$**	**$2.66x10^2$**	**$2.6x10^2$**	**$8.3x10^1$**
	(Range)	ND (10^2)- 1.0x104	ND (10^3)	$8.02x10^2$- $3.9x10^3$	ND (10^2)- $2.1x10^3$	ND (10^2)- $1.0x10^3$	ND (10)²- $8.0x10^2$	ND (10^2)- $1.3x10^3$	ND (10^2)- $2.0x10^2$
25	Total Count	**$1.7x10^4$**	**$9.48x10^3$**	**$2.56x10^3$**	**$1.95x10^3$**	**$6.8x10^2$**	**$7.5x10^2$**	**$6.2x10^2$**	**$6.0x10^2$**
	(Range)	ND (10^2)- $2.3x10^6$	ND (10^2)- $2.6x10^6$	$1.0x10^2$- $3.5x10^4$	$1.0x10^2$- $4.1x10^4$	ND (10^2)- $7.3x10^3$	ND (10^2)- $6.9x10^3$	ND (10^2)- $6.1x10^3$	ND (10^2)- $5.6x10^3$
	Bl (-Bc)	**$3.0x10^5$**	**$3.3x10^5$**	**$5.45x10^3$**	**$4.8x10^3$**	**$7.2x10^2$**	**$8.3x10^2$**	**$7.1x10^2$**	**$6.5x10^2$**
	(Range)	ND (10^2)- $2.3x10^6$	ND (10^2)- $2.6x10^6$	ND (10^2)- $3.5x10^4$	ND (10^2)- $4.1x10^4$	ND (10^2)- $7.3x10^3$	ND (10^2)- $6.9x10^3$	ND (10^2)- $6.1x10^3$	ND (10^2)- $5.6x10^3$
	Bl (+Bc)	**$3.1x10^3$**	**$1.8x10^3$**	**$2.0x10^3$**	**$2.1x10^3$**	**$6.5x10^2$**	**$6.5x10^2$**	**$6.02x10^2$**	**$7.0x10^2$**
	(Range)	ND (10^2)- $3.2x10^4$	ND (10^2)- $1.6x10^4$	ND (10^2)- $3.5x10^4$	ND (10^2)- $2.5x10^4$	ND (10^2)- $3.8x10^3$	ND (10^2)- $4.3x10^3$	ND (10^2)- $3.3x10^3$	ND (10^2)- $3.5x10^3$
20	Total Count	**$8.1x10^2$**	**$9.25x10^2$**	**$5.1x10^2$**	**$4.75x10^2$**	**$4.3x10^2$**	**$3.6x10^2$**	**$4.6x10^2$**	**$4.5x10^2$**
≤ 10	Total Count	**$5.1x10^3$**	**$4.6x10^3$**	**$4.4x10^3$**	**$4.1x10^3$**	**$5.0x10^3$**	**$3.85x10^3$**	**$4.0x10^3$**	**$5.5x10^3$**

¶ Mean Total count for *B. licheniformis* § Mean Total count for *B. licheniformis* when not competing with *B. cereus*

¥ Mean Total count for *B. licheniformis* when in direct competition with *B. cereus*

Table 5.14 (a) illustrate the number of reconstituted IMF samples (prepared at 56°C and/or 90°C) supporting the growth of *B. cereus I* at different concentration levels (CFU g^{-1}) over a 24 hour period at temperatures ≥25°C.

Storage Temp (°C)	Time (hrs)	Number of IMF samples supporting growth of B. cereus I (CFU g^{-1}) after reconstitution at 56 and 90°C														Total No. (n)
		≤ 10^2		≤ 10^3		≤ 10^4		> 10^4		≥ 10^5		≥10^6		≥ 10^7		
		58°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	
35°C	0	10	9	-	1	-	-	-	-	-	-	-	-	-	-	10
	8	4	4	4	4	2	2	-	-	-	-	-	-	-	-	10
	14	1	2	3	2	5	1	1	5	-	-	-	-	-	-	10
	24	2	-	-	-	-	-	-	-	2	-	1	1	7	7	10
25°C	0	16	18	2	-	-	-	-	-	-	-	-	-	-	-	18
	8	17	16	1	2	-	-	-	-	-	-	-	-	-	-	18
	14	14	13	3	4	1	1	-	-	-	-	-	-	-	-	18
	24	1	-	-	1	2	1	5	6	7	5	1	4	2	1	18

Table 5.14 (b) illustrate the number of reconstituted IMF samples (prepared at 56°C and/or 90°C) supporting the growth of *B. cereus II* at different concentration levels (CFU g^{-1}) over a 24 hour period at temperatures ≥25°C.

Storage Temp (°C)	Time (hrs)	Number of IMF samples supporting growth of B. cereus II (CFU g^{-1}) after reconstitution at 56 and 90°C														Total No. (n)
		≤ 10^2		≤ 10^3		≤ 10^4		> 10^4		≥ 10^5		≥10^6		≥ 10^7		
		58°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	56°C	90°C	
35°C	0	13	8	-	5	-	-	-	-	-	-	-	-	-	-	13
	8	8	4	2	5	3	4	-	-	-	-	-	-	-	-	13
	14	2	-	5	6	4	3	2	4	-	-	-	-	-	-	13
	24	1	-	-	-	-	-	-	-	1	-	2	4	9	9	13
25°C	0	24	26	2	-	-	-	-	-	-	-	-	-	-	-	26
	8	23	22	3	4	-	-	-	-	-	-	-	-	-	-	26
	14	4	2	22	23	-	1	-	-	-	-	-	-	-	-	26
	24	2	1	-	-	-	2	6	3	13	16	4	4	1	-	26

Table 5.15 illustrates the strains of *B. cereus II* capable of producing diarrhoeal enterotoxin and the potential for these organisms to grow at psychrotrophic temperatures in IMF samples.

IMF Product	Code No.	Date of Isolation	Diarrhoegenic Toxin Produced	Growth at		
				5°C	8°C	30°C
SMA White Cap	NR2	29-09-93	-	+	+	+
Nutrilon Plus	NR3	29-09-93	+	+	+	+
Milupa Aptamil	NR8	29-09-93	-	-	-	+
NCTC 11145 (Control)	No.9		+	-	-	+
Nutrilon Premium	NR11	07-04-93	+	-	+	+
Nutrilon Plus	NR14	01-07-93	-	-	-	+
SMA Gold Cap	NR19	30-06-93	-	-	-	+
Nutrilon Premium	NR27	15-06-93	-	-	+	+
Farleys Oster Milk Two	NR28	15-07-93	-	-	+	+
Milupa Milumil	NR30	31-03-93	+	-	+	+
Milupa Milumil	NR36	11-05-93	+	-	-	+
SMA Gold Cap	NR37	08-09-93	-	-	-	+
Farleys Oster Milk Two	NR38	29-10-93	-	-	+	+
Farleys Oster Milk Two	NR39	08-09-93	-	-	-	+
SMA White Cap	NR40	14-09-93	-	-	-	+
Milupa Milumil	NR41	29-10-93	-	-	-	+
Nutrilon Plus	NR46	12-09-93	+	-	-	+
Milupa Aptamil	NR48	20-10-93	-	-	+	+
Farleys Oster Milk One	NR52	29-10-93	-	-	-	+
Nutrilon Plus	NR53	12-10-93	+	-	-	+
SMA Gold Cap	NR57	14-09-93	-	-	-	+
Milupa Milumil	NR59	14-09-93	-	-	+	+
Milupa Aptamil	NR61	29-10-93	-	-	-	+
Nutrilon Premium	NR62	29-10-93	+	-	+	+
Farleys Oster Milk One	NR70	14-12-93	-	-	+	+
Nutrilon Plus	NR89	23-07-93	-	-	-	+
SMA Gold Cap	NR91	23-07-93	-	-	-	+
Milupa Aptamil	NR92	05-08-93	-	-	-	+
SMA Gold Cap	NR93	05-05-93	-	-	-	+
Nutrilon Plus	NR99	11-11-93	-	+	+	+
Nutrilon Premium	NR100	11-11-93	-	-	-	+
Farleys Oster Milk Two	NR108	20-10-93	-	-	+	+
Farleys Oster Milk Two	NR109	15-02-94	+	-	-	+
SMA Gold Cap	NR110	17-02-94	-	-	-	+
Farleys Oster Milk Two	NR113	17-02-94	-	-	-	+
Farleys First Milk	NR114	17-02-94	-	+	+	+
Nutrilon Premium	NR123	09-01-94	-	-	+	+
Farleys Second Milk	NR125	15-01-94	+	-	+	+
Farleys Oster Milk One	NR126	15-01-94	-	-	+	+

5.3 Diarrhoeal enterotoxin production by *Bacillus* species in infant milk formula.

Baby foods are considered a food class of high risk due to the high susceptibility of the consumer population to enteric pathogens, severe response to toxins and the resulting increased mortality. However, there is no requirement for these products to be sterile (ICMSF 1986). Although infant milk formula undergoes stringent heat treatments, microbiological analysis of these commercially available products have shown that they can contain $>10^2$ aerobic spore forming bacilli g^{-1} (sections 2.3.2 and 5.2).

While it has been documented that raw milk is both a poor germination and toxin producing medium for *B. cereus* (i.e. due to the low level of free amino acids, no readily utilisable carbon source and low oxygen concentration), reconstituted infant milk formula is theoretically a better germination medium as the temperatures produced during the spray drying process and during subsequent reconstitution of the powder may result in the heat germination of spores (section 2.2.4). Indeed, *B. cereus* spores are capable of germinating without preliminary heat treatment, however, the rate of germination and the proportion of spores are higher when they have been previously subjected to a lethal heat treatment (e.g. 60°C for 1 hour). Although infant milk formula and milk have been frequently cited as a potential source of *B. cereus* and other aerobic spore forming bacilli (section 2.3.2), very few reported incidents of food borne illness has been attributed to these food products (2.3.3). Factors such as low oxygen concentration and the absence of glucose in reconstituted IMF and milk have been postulated as the reason for this low level of food related illness (section 2.2.6).

Research has shown that besides glucose, starch is also a good carbon source for both growth and toxin production (section 2.2.6). While starch is not normally added to infant milk formula as a supplement, maltodextrin (a derivative of hydrolysed starch), has been known to be added to certain brands of follow-on infant milk formula. Maltodextrin is derived from the spray drying of a purified aqueous solution of nutritive carbohydrates which were obtained by the enzyme hydrolysis of starch. It is

used as a supplement in the manufacture of infant milk formula and consists of dextrose (1%), maltose (5%), maltotriose (8.5%) and higher sugars (85.5%) (Cerestar 1994).

The aim of this series of experiments was to determine whether a variety of infant milk formula could support the production of diarrhoeal enterotoxin and to establish whether or not the supplementation of these formulae with maltodextrin (i.e. the grade used in the manufacture of baby foods) would either permit and/or enhance enterotoxin production by known diarrhoegenic strains of *Bacillus cereus*.

5.3.1 The production of the diarrhoeal enterotoxin by enterotoxigenic strains of *B. cereus* cultivated in TSYEB with and without a supplement of 3.8% Maltodextrin.

In this study 4 strains of *B. cereus* (i.e. 3 strains isolated from IMF and a NCTC 11145 positive control for toxin production) previously shown to produce the diarrhoeal enterotoxin (section 5.2) were inoculated into TSYEB and/or TSYEB supplemented with 3.8% maltodextrin (section 3.4.1). A percentage of 3.8 was selected as this reflected the amount of maltodextrin added to certain commercial infant milk formulae (e.g. Farleys Second Milk). The strains were cultivated under conditions known to be favourable for diarrhoeal toxin production (section 3.9) and the test culture media were then analysed for the presence of toxin via the *Bacillus cereus* Enterotoxin-Reverse Phase Latex Agglutination test kit (section 3.9).

The enterotoxin assay was initiated by streaking the 3 strains of *Bacillus cereus* type 11 (i.e. numbers 3, 11, and 62 previously recovered from infant milk formulae and known to produce the diarrhoeal enterotoxin) to single colonies on TSYEA plates. The plates were incubated for 24 hr at 35°C. In addition, single colonies of *B. cereus* NCTC 11145 and *B. sphaericus* NCTC 7582 (positive and negative controls for enterotoxin production respectively) were also obtained on TSYEA plates as described above. The identity of the test *Bacillus* spp. was confirmed by carrying out

an API 50CHB/API 20 E reaction profile supplemented with the other key morphological and/or biochemical tests described in section 5.1.

The test cultures were inoculated into duplicate 250 ml Erlenmyer flasks containing 100 ml of either TSYEB or TSYEB supplemented with 3.8% maltodextrin (see section 3.4.1). A sugar assay was carried out on a 3.8% maltodextrin solution before and after filtration to determine whether all the maltodextrin had passed through the 0.45μm membrane filter. The result of this showed that 1.163g glucose L^{-1} and 1.193g glucose L^{-1} were present before and after filtration respectively and inferred that all the maltodextrin was successfully delivered into the filtrate.

The test cultures were then incubated at 32°C for 18 hours under gyrotary conditions at 150 rpm. After incubation, 1 ml aliquots of the test culture was aseptically transferred to duplicate 1.5 ml Eppendorf tubes and centrifuged at 11,500g for 10 mins at 4°C (section 3.7.1). The non-turbid, fat free supernatant was aseptically aspirated into a sterile 1.5 ml Eppendorf tube and retained for analysis of toxin content. Duplicate 25μl aliquots of each test culture supernatant were then analysed for diarrhoeal toxin content via the Reverse Phase Latex Agglutination test kit (section 3.9).

The result of this enterotoxin analysis illustrated that all four strains of *B. cereus* (the 3 wild type and 1 reference strain) were capable of producing the diarrhoeal enterotoxin in both TSYEB and TSYEB which had been supplemented with 3.8% Maltodextrin. This was reflected in a positive latex agglutination reaction in all the sample wells down to well "G" (the 7th well). The RPLA test system failed to detect any enterotoxin from the *B. sphaericus* test culture control organism. The latex control wells remained negative for toxin production. The study illustrated that, due to TSEYB's ability to support toxin production, it was therefore an unsuitable control medium for investigating the possible link between maltodextrin content and diarrhoeal enterotoxin production. The mean *B. cereus* cell concentration at the point of toxin analysis was approximately 2.0×10^8 CFU ml.

5.3.2 Production of the diarrhoeal enterotoxin by enterotoxigenic strains of *B. cereus* cultivated in a Basal Synthetic Medium supplemented with 3.8% Maltodextrin.

Considering that TSYEB supported growth and toxin production (irrespective whether or not maltodextin was added), the study was repeated using a Basal Synthetic Medium (section 3.3.2.3). Single colonies were obtained on TSYEA plates as described in section 5.3.1. These test cultures were then inoculated into a duplicate set of 250 ml Erlenmyer flasks containing 100 ml Basal Medium and Basal Medium which was supplemented with 3 8% maltodextrin and then incubated at 32°C for 18 hours at 150 rpm. After incubation, the test cultures were analysed for diarrhoeal enterotoxin production via the RPLA system as described in section 3.9.

The result of this enterotoxin analysis revealed that all four strains of *B. cereus* exhibited toxin production in basal medium which had been supplemented with 3.8% maltodextrin, whereas enterotoxin was not detected in either the supernatants from the Basal Media which had not been supplemented with maltodextrin or from the test media which had been cultured *with B. sphaericus*. Although the RPLA test system should not be employed to quantitatively measure the level of toxin present from each test culture, the enterotoxin produced by *B. cereus* (previously cultivated in 3.8% maltodextrin basal media) did produce a positive latex agglutination reaction beyond well "C" (3rd well) of the microtitre plates (Table 5.16). However, by virtue of the fact that double dilutions of enterotoxin were made in each of the wells down as far as the 7th well, it would appear that the 3.8% maltodextrin basal medium (i.e. toxin detected in 3rd well) was an inferior medium for the production of enterotoxin compared to growth and toxin production in TSYEB (i.e. toxin detected in the 7th well, section 5.3.1).

Although enterotoxin was produced by toxigenic *B. cereus* strains in Basal medium supplemented with 3 8% Maltodextrin, this does not necessarily imply that IMF (containing the same concentration of maltodextrin) would support toxin production.

Table 5.16 Production of diarrhoeal enterotoxin by *B. cereus* (number 11) in either TSYEB or a Basal Medium which had been supplemented with 3.8% Maltodextrin.

Well No. on Microtitre Plate[d]	Bacillus Cereus Enterotoxin -Reverse Phase Latex Agglutination reaction					
	Basal Medium[a] (3.8% Maltodextrin)		TSYEB (3.8% Maltodextrin)		Basal Medium (0% Maltodextrin)	
	Sensitised[b]	Control[c]	Sensitised	Control	Sensitised	Control
1st	+	-	+	-	-	-
2nd	+	-	+	-	-	-
3rd	+	-	+	-	-	-
4th	-	-	+	-	-	-
5th	-	-	+	-	-	-
6th	-	-	+	-	-	-
7th	-	-	+	-	-	-
8th (Control)	-	-	-	-	-	-

[a] Type of cultivation medium
[b] Represents the first reaction column consisting of 8 V-shaped wells containing latex sensitised with specific B. cereus anti-enterotoxin (the 8th well contains buffer and no sample).
[c] Represents the second reaction column consisting of 8 wells containing latex suspension sensitised with non-immune rabbit globulins (i.e. Latex control wells)
[d] Represents the well position or number on the microtitre plate where a latex agglutination reaction occurred (designated as +), as a double dilution was carried out in each well, the 1st well contains the largest amount of sample.

5.3.3 Detection of enterotoxin in infant milk formulae containing Maltodextin using a fat free culture extract (via the Filtron® Stirred Cell Ultrafiltration System).

The aim of this study was to determine whether *B. cereus* diarrhoeal enterotoxin could be produced and detected in reconstituted infant milk formulae (which naturally contained or was supplemented with Maltodextrin). In order to successfully assay cultured IMF products for enterotoxin content, it was necessary to separate the fat content from the cultured IMF sample as this is a prerequisite for the proper operation of the RPLA test system. *Bacillus cereus* produces the multicomponent diarrhoeal enterotoxin (molecular weight greater than 100 kDa) in the late exponential phase of growth, which consists of 3 molecular weight subunits, namely 38, 39.5 and 45 k Da (section 2.2.6). The 39.5 kDa component has been specifically implicated in the diarrhoeal type syndrome. The aim of this study was to determine whether or not the

Filtron® Stirred Cell Ultrafiltration Separation System could successfully separate the enterotoxin (either as the complete multicomponent toxin or in the sub unit form) from the other constituents present in infant milk formula (section 3.10) and to challenge the efficacy of various IMF products in their ability to support growth and enterotoxin production.

The study was initiated by inoculating a selection of known *B. cereus* type 11 toxin producers (i.e. numbers 11, 42) into a series of duplicate 250 ml Erlenmyer flasks containing 100 ml of the following infant milk formula (section 3.5.2):

a) reconstituted Farley's Second Milk which contained maltodextrin (3.78%) and lactose (4.8%) and had been tyndallised to sterility (**FSM**).
b) reconstituted Cow & Gate Premium which contained lactose (7.2%) and was reconstituted at 56°C in sterile distilled water (**C%GM**).
c) reconstituted Cow & Gate Premium which contained lactose (7.2%) and was supplemented with 3.78% maltodextrin, and was reconstituted at 56°C in sterile distilled water [**C%GM (MD)**].
d) reconstituted Farley's Second Milk which contained maltodextrin (3.78%) and lactose (4.8%) and was reconstituted at 56°C in sterile distilled water [**FSM (MD)**].
e) reconstituted Farley's Follow on Milk which contained maltodextrin (8%) and was reconstituted at 56°C in sterile distilled water (**FFOM**).

These infant milk formulae were tyndallised (section 3.13) and/or simply reconstituted at a water temperature of 56°C in sterile distilled water in order to ensure that limited nutrient destruction of heat labile constituents occurred. Supplementation of these infant milk formula with the respective percentages of maltodextrin (e.g. 8% and 3.78% for Farley's Follow On and Second Milks respectively) was achieved by aseptically transferring the required volume (ml) of 10% maltodextrin stock solution to each of the IMF under study (section 3.4.1). These test culture flasks were then incubated for 18 hours at 32°C under gyrotary conditions (0 rpm). A total aerobic mesophilic count (CFU ml^{-1}) was carried out (on TSYEA and LSA plates) on each test culture prior to extraction of enterotoxin.

Afterward, a 2 ml aliquot of the test culture was ultrafiltrated through a Filtron™ Stirred Cell Concentrator which had been fitted with an Omega 300 kDa nominal molecular weight cut off polyethersulfone membrane (section 3.10.2). As ultrafiltration is a method whereby biomolecules or cells are separated/concentrated based on molecular weight of pore size exclusion, biomolecules with a pore size diameter greater than 300 kDa were retained by the membrane (i.e. enterotoxin with a molecular weight cut off less than this threshold value passed through). The sample test culture (2ml) was transferred to the disposable cell unit and nitrogen gas regulated up to 20 psi provided the driving force for the filtration. A laboratory magnetic stir plate activated the fitted stir bar creating a gentle turbulence at the membrane surface. This caused the macromolecules (>300 kDa) to be swept off the membrane surface and the micromolecules (<300Kda) to pass through. The concentrate and the filtrate were retained for enterotoxin analysis via the RPLA system (section 3.9).

The concentrate was retained and assayed for toxin content in order to establish whether or not all the enterotoxin had passed through the membrane (section 3.9). This was achieved by resuspending the concentrate in 2 ml 0.01M PBS and then centrifuging a 1 ml aliquot at 11,500g for 10 mins at 4°C. The middle cell free liquid section between the upper fat layer and the lower bacterial mass layer was aseptically aspirated and analysed for enterotoxin content as described in section 3.9. Microcentrifugation of the concentrate did not guarantee a non-turbid, fat free cell culture extract (section 3.7.1), and this was the reason for employing the ultrafiltration system for the cultured IMF samples.

The filtrate and the cell free liquid aspirate (resulting from centrifugation of resuspended concentrate) were filter sterilised through a 0.45μm Millipore membrane prior to enterotoxin analysis (as recommended by the manufacturer of the BCET-RPLA system, section 3.9).

The results of this extraction illustrated that while lactose permitted the growth and proliferation of *B. cereus*, it did not allow the subsequent production of the diarrhoeal toxin (Tables 5.17 and 5.18). However, infant milk formulae containing maltodextrin (either naturally present in formulation or supplemented) permitted

growth of *B. cereus* (No 11 and No 42) and subsequent toxin production as detected via the latex agglutination reaction to the 7th well. Infant formulae which had been initially cultivated by *B. sphaericus* NCTC 7582 failed to produce any toxin.

Examination of Table 5.17 revealed that the filtrate provided the highest levels of enterotoxin agglutination reactions (7th well), indicating that this protein based enterotoxin was successfully produced in infant milk formulae and recovered via ultrafiltration (using a 300 kDA MWCO membrane). The latex control wells (containing sample and a latex suspension which had been sensitised with non-immune rabbit globulins) remained negative for the 3 species of *Bacillus* under investigation. The cell free liquid aspirate which had been removed from the centrifuged resuspended concentrate exhibited positive latex agglutination reactions via the BCET-RPLA test kit. This would possibly suggest that not all the enterotoxin had passed through the membrane and that it had mixed with the other macromolecules in the concentrate. Also that aseptic aspiration of the cell free liquid section (position between the upper fat layer and lower casein-bacterial mass layer) produced as a result of centrifugation of the concentrate was sufficient to provide a non-turbid, fat free extract for subsequent enterotoxin analysis. The RPLA reactions shown in Tables 5.17 and 5.18 are the result of the average readings from duplicate test culture sample microtitre reaction plates.

Table 5.17 BCET-RPLA reaction profile for *B. cereus II* (No 11) which had been cultivated in a variety of infant milk formula with and without maltodextrin supplementation.

V-Well N°	Bacillus Cereus Enterotoxin-Revrse Phase Latex Agglutination reaction									
	FSM[a]		C&GM		C&GM(MD)		FSM (MD)		FFOM	
	Filt†	Conc‡	Filt	Conc	Filt	Conc	Filt	Conc	Filt	Conc
1st	+	+	-	-	+	+	+	+	+	+
2nd	+	+	-	-	+	+	+	+	+	+
3rd	+	+	-	-	+	-	+	+	+	+
4th	+	+	-	-	+	-	+	-	+	+
5th	+	-	-	-	+	-	+	-	+	+
6th	+	-	-	-	+	-	+	-	+	-
7th	+	-	-	-	-	-	+	-	+	-
Control	-	-	-	-	-	-	-	-	-	-

† Refers to filtrate samples (where + illustrates a positive agglutination reaction and -, a negative reaction).
‡ Refers to the concentrate (where + illustrates a positive agglutination reaction and -, a negative reaction).
a Refers to the type of culture media which can be identified from the text.

Table 5.18 BCET-RPLA reaction profile for *B. cereus II* (No 42 or NCTC 11145) which had been cultivated in a variety of infant milk formula with and without maltodextrin supplementation.

V-Well N°	Bacillus Cereus Enterotoxin-Revrse Phase Latex Agglutination reaction									
	FSM[a]		C&GM		C&GM(MD)		FSM (MD)		FFOM	
	Filt†	Conc‡	Filt	Conc	Filt	Conc	Filt	Conc	Filt	Conc
1st	+	+	-	-	+	+	+	+	+	+
2nd	+	+	-	-	+	+	+	+	+	+
3rd	+	+	-	-	+	+	+	+	+	+
4th	+	-	-	-	+	-	+	-	+	+
5th	+	-	-	-	+	-	+	-	+	+
6th	+	-	-	-	+	-	+	-	+	-
7th	+	-	-	-	+	-	+	-	+	-
Control	-	-	-	-	-	-	-	-	-	-

† Refers to filtrate samples (where + illustrates a positive agglutination reaction and -, a negative reaction).
‡ Refers to the concentrate (where + illustrates a positive agglutination reaction and -, a negative reaction).
[a] Refers to the type of culture media which can be identified from the text.

5.3.4 The effect of maltodextrin, glucose and lactose concentration on subsequent diarrhoeal enterotoxin production by *Bacillus* species.

The aim of this study was to establish whether the supplementation of Basal Media with different concentrations of maltodextrin, glucose or lactose would influence diarrhoeal enterotoxin production. It has been established that infant milk formulae containing ≥3.78g maltodextrin 100 ml^{-1} reconstituted IMF does enable enterotoxigenic strains of *B. cereus* to produce the diarrhoeal toxin (section 5.3.2). In addition, this study was designed to identify the percentage of maltodextrin or glucose required to initiated toxin production, and to determine whether lactose (a natural source of carbohydrate in milk) would permit any growth of *B. cereus* and/or subsequent toxin production in the basal synthetic medium. A study was also carried out in order to establish whether cultivation of the Maltodextrin-Basal medium with a culture of *B. licheniformis* for 8 hours prior to the addition of enterotoxigenic *B. cereus* would influence the level of enterotoxin produced.

A 100 ml basal medium was prepared and sterilised (section 3.3.2.3) in a series of 250 ml Erlenmeyer flasks. Stock solutions of 10% maltodextrin, 10% glucose and 10% lactose were filter sterilised separately in a Nalgene® Membrane Filtration System which had been fitted with a sterile 0.45μm Millipore membrane.

A defined volume (ml) of the 10% stock maltodextrin solution (i.e. 0.1 ml, 1 ml, 10 ml and 38 ml) was aseptically added to a duplicate set of sterilised basal medium (i.e. containing 99.9 ml, 99 ml, 90 ml and 62 ml Basal medium respectively) resulting in a working maltodextrin basal medium of 0.01%, 0.1%, 1.0% and 3.8% maltodextrin. The same principle was applied to the 10% stock glucose solution to provide a 0.1% and 3.0% Glucose Basal Medium and to the 10% stock lactose solution to provide a 0.1, 1.0 and 3.0 % lactose Basal Medium.

Two enterotoxigenic strains of *Bacillus cereus* (No. 11, isolated from infant milk formula and No. 42 , a reference strain from the National Collection of Typed Cultures- number 11145) and two non-diarrhoeal enterotoxin producers (*B. sphaericus* NCTC 7582 and *B. licheniformis* NCTC 10341) were streaked to single

colonies on TSYEA plates (37°C for 24 hours). The four test organism were inoculated into separate 250 ml Erlenmeyer flasks containing 100 ml TSYEB and then cultivated at 35°C for 24 hours at 110 rpm. The sub culture media (1 ml aliquots) were centrifuged, washed and resuspended in 0.01 PBS. A decimal serial dilution of the resuspended PBS culture was aseptically carried out down to 10^{-4}. A 1 ml aliquot of the *B. licheniformis* 10^{-4} dilution was inoculated into the above series of basal media containing either maltodextrin (0.01, 0.1, 1.0 and 3.78%), glucose (0.1, 3.0%) or lactose (0.1, 3.0%) and was cultivated for 9 hours at 32°C under orbital conditions (250 rpm).

After the 8 hours cultivation, a 1 ml aliquot (of the 10-4 dilution) of the test organism (either *B. cereus* No. 11, *B. cereus* No 42, or *B. sphaericus*) was inoculated into either: (a) the *B. licheniformis* precultured flasks; (b) a sterile set of basal media containing the designated concentrations of maltodextrin, glucose or lactose. Both sets of flasks were incubated under conditions favourable for toxin production, i.e. 32°C for 19 hours at 250 rpm.

In addition to the total aerobic mesophilic count (CFU ml^{-1} -on BCSA and TSA plates) carried out on each flask at the point of inoculation, a count was also performed on the *B. sphaericus* negative enterotoxin control, the basal media control (for each carbon source) and a 19 hour test culture control of *B. licheniformis* were carried out. After the 19 hours (for test media inoculated with one test organism) and 28 hours (for test media initially inoculated with *B. licheniformis* and then the respective test organism) incubation period, a 1.0 ml aliquot from each test culture was aseptically transferred to a 1.5 ml Eppendorf tube and centrifuged at 11,500 for 10 mins at 4°C (section 3.7.1). A non-turbid, fat free supernatant was obtained and subsequently analysed for diarrhoeal enterotoxin via the Bacillus Cereus Enterotoxin-Reverse Phase Latex Agglutination (BCET-RPLA) test kit (section 3.9).

The results from the duplicate enterotoxin assays for *B. cereus* No. 11 (isolated from IMF) and No. 42 (NCTC 11145 control) which had been cultivated with or without *B. licheniformis* were averaged and presented in Table 5.19. In this table, the appearance of a positive agglutination reaction in the lowest dilution well (i.e. double dilutions of the sample were made in each well down to the 7th well) was shown in

addition to the total aerobic mesophilic count (CFU ml^{-1}) for the undiluted test culture.

Table 5.19 Production of diarrhoegenic enterotoxin by of two strains of *B. cereus II* in various basal media containing different carbon sources via the BCET-RPLA test system.

Basal Medium	BCET-RPLA reaction†				Total Aerobic Mesophilic Count (CFU ml^{-1})			
	With B. licheniformis‡		Without B. licheniformis		With B. licheniformis		Without B.licheniformis	
	No.11[a]	No.42[b]	No.11	No.42	No.11	No.42	No.11	No.42
3.78% Maltodextrin	2nd	2nd	2nd	2nd	$7.0x10^6$	$1.07x10^7$	$1.25x10^7$	$1.30x10^7$
1.0% Maltodextrin	5th	5th	5th	5th	$1.63x10^7$	$1.57x10^7$	$1.33x10^7$	$1.41x10^7$
0.1% Maltodextrin	-	2nd	-	-	$7.4x10^6$	$1.09x10^7$	$9.8x10^6$	$1.11x10^7$
0.01% Maltodextrin	-	-	-	-	$6.1x10^6$	$8.5x10^6$	$7.3x10^6$	$7.9x10^6$
3.0% Lactose	-	-	-	-	$9.2x10^6$	$1.03x10^7$	$1.15x10^7$	$9.6x10^6$
1.0% Lactose	-	-	-	-	$8.3x10^6$	$9.6x10^6$	$1.05x10^7$	$8.6x10^6$
3.0% Glucose	2nd	3rd	2nd	2nd	$1.82x10^7$	$2.3x10^7$	$2.05x10^7$	$2.11x10^7$
1.0% Glucose	5th	5th	5th	5th	$1.96x10^7$	$1.5x10^7$	$1.81x10^7$	$2.01x10^7$
No Carbohydrate added	-	-	-	-	$4.8x10^2$	$3.95x10^2$	$6.1x10^2$	$6.3x10^2$

† The RPLA reaction profile, where the lowest dilution well where a positive latex agglutination reaction occurred is illustrated . The minus symbol designates a negative RPLA reaction.
‡ Where the test medium was initially inhabited by a 9 hour B. licheniformis cell culture prior to the addition of the test organism.
[a] B. cereus which had been isolated from an infant milk formula and a known enterotoxigenic producer.
[b] B. cereus NCTC 11145,a reference strain for positive diarrhoeal toxin production.

Examination of the information presented in Table 5.19 showed that under culture conditions where *B. cereus 11* was the only organism in a basal medium containing 1.0% maltodextrin, this test medium would support both growth and toxin

production. However, it did appear that both *B. cereus* test strains were capable to producing enterotoxin in basal media supplemented with a lower concentration of maltodextrin (0.1%) when the test medium has been initially cultivated for 9 hours with a strain of *B. licheniformis* prior to the addition of *B. cereus 11*.

Growth of *B. cereus* and *B. licheniformis* in the same 1% glucose medium resulted in the production of enterotoxin (as detected by a positive latex agglutination reaction in the 5th well). However, *B. cereus* either produced toxin at a reduced level or failed to produce toxin when cultivated with or without *B. licheniformis* respectively in a 0.1% maltodextrin Basal medium. Although a certain level of maltodextrin appeared to be required for the formation of this toxin (i.e. 0.1%), the supplementation of this basal medium with greater concentrations of maltodextrin did not enhance enterotoxin production (P0.05). This was illustrated by *B. cereus* (number 11) producing a positive latex agglutination reaction in the 5th and 2nd wells on the microtitre plates when cultivated in a basal medium containing 1.0% and 3.0% glucose respectively.

While lactose which had been added to the basal medium at a concentration of >0.1g 100 ml^{-1} permitted growth of *B. cereus*, this carbon source did not support diarrhoeal toxin production. Both *B. sphaericus* and *B. licheniformis* failed to produce toxin in any of the test media and the latex control wells for the *B. cereus* test cultures were shown to be negative for toxin formation. A table of microbial growth and RPLA test reactions was not constructed for the above *Bacillus* species (i.e. employed as negative controls) as all the sample wells in the respective microtitre plates exhibited a negative reaction for toxin formation.

The results of this study revealed that diarrhoeal enterotoxin can be produced by certain enterotoxigenic strains of *B. cereus* in infant milk formula which contains maltodextrin at levels ≥ 0.1% and lactose was a poor carbon source for enterotoxin production.

5.3.5 Recovery of the diarrhoeal enterotoxin from infant milk formula containing different levels of maltodextrin via the Intersep® ultracentrifugation technique.

The objective of this study was to determine whether the toxin produced by enterotoxigenic strains of *B.* cereus could be recovered from infant milk formula by using an alternative fat separation technique (i.e. Intersep® Filtration System) to that of ultrafiltration using a similar experimental procedure to that described in section 5.3.4.

The enterotoxigenic *B. cereus* strains No. 11 (an IMF isolate) and No. 42 (NCTC 11145) were streaked to single colonies as described above. The *B. sphaericus* (NCTC 7582.) and *B. licheniformis* (NCTC 10341) were used as test controls. A single colony from each of the overnight TSYEA plate was aseptically inoculated into a separate 250 ml Erlenmeyer flask containing 100 ml of TSYEB and then cultivated at 35°C for 24 hours at 250 rpm. After incubation, the four test organisms were washed and resuspended in 1.0 ml PBS (in a 1.5 ml Eppendorf tube) as described earlier. A decimal dilution was carried out on all the resuspended test cultures and a 1 ml aliquot of the *B. licheniformis* 10^{-4} dilution was inoculated into a series of separate 250 ml Erlenmeyer flasks containing 100 ml of tyndallised infant milk formula (section 3.5.2). The composition of these test media was as follows:

a) Farleys Second Milk containing 3.78% maltodextrin (**FSM**)
b) Cow & Gate Premium and not containing any maltodextrin (**C&GPrm**)
c) Cow & Gate Premium aseptically supplemented with 1% filter sterilised maltodextrin (**C&GPrm-Md**).
d) Basal Synthetic Medium aseptically supplemented with 1% filter sterilised maltodextrin (**BM-Md**).
e) Basal Synthetic Medium Control not containing any carbon source (**BM**).

The test media containing the 1 ml of *B. licheniformis* resuspended test culture, was incubated for 9 hours at 32°C under gyrotary conditions of 250 rpm as described in section 5.3.4. After 9 hours cultivation of the homogenous *B. licheniformis* cells, a 1 ml aliquot (of the 10^{-4} dilution) of the test organism (either *B. cereus* No. 11, *B. cereus* No 42, or *B. sphaericus*) was inoculated into either: (a) the above *B. licheniformis* precultured flasks; or (b) a complimentary set of sterile cultivation media. Both sets of flasks were incubated under conditions favourable for toxin

production, i.e. 32°C for 19 hours at 250 rpm. A total aerobic mesophilic count (CFU ml^{-1}-on BCSA and TSA plates) was carried out on each flask at the time of inoculation.

After 19 hours (for test media which had been inoculated with just *B. cereus* or *B. sphaericus*) and 28 hours (for test media initially cultivated for 9 hours with *B. licheniformis* and then the respective test organism for an additional 19 hours) incubation, 5 ml aliquots were aseptically transferred to the filter tube reservoir of an Intersep® Filtration System which had been fitted with a 100 kDa PES membrane. The sample was then centrifuged (section 3.7.2) at 16,000g for 15mins at 4°C according to the instruction manual for "Intersep Bio Concentrators" (section 3.11). The filtrate was retained and analysed for the presence of enterotoxin (section 3.9). The concentrate was resuspended in 0.01M PBS and a 1 ml aliquot was centrifuged in a microcentrifuge at 11,500g for 10 mins at 4°C (section 3.7.1).

Controls were carried out on each basal medium (containing the different carbon source), a negative control on the RPLA system using a member of the *Bacillus* species (*B. sphaericus*) and a 19 hour test *B. licheniformis* culture control was analysed for enterotoxin production.

Examination of the information presented in Table 5.20 revealed that ultracentrifugation of test cultures through the Intersep Bio-Concentrator successfully provided a non turbid, fat free extract suitable for subsequent enterotoxin analysis via the BCET-RPLA test system. *B. cereus 11* (No. 11) demonstrated toxin production in both infant formula and basal media which contained maltodextrin. Indeed, the enterotoxin was detected in the filtrate and the concentrate from samples taken from infant milk formula. This would possibly suggest that not all the enterotoxin components eliciting a positive latex agglutination reaction passed through the 100 kDa molecular weight cut off membrane and that some of this toxin was retained in the IMF concentrate. Microcentrifugation of the resuspended concentrate (from infant milk formula containing maltodextrin) provided an extract suitable for enterotoxin analysis.

Table 5.20 Detection of diarrhoeal enterotoxin in the filtrate and/or concentrate of a variety of test media previously cultured with *B. cereus II* (No. 11) via the BCET-RPLA test system.

Test medium	BCET-RPLA reaction†				Total Aerobic Mesophilic Count (CFU ml^{-1})			
	With‡ B. licheniformis		Without B. licheniformis		With B. licheniformis		Without B.licheniformis	
	Filtrate[b]	Concentrate[c]	Filtrate	Concentrate	Filtrate	Concentrate	Filtrate	Concentrate
FSM[a] (3.78% Maltodextrin)	6th	5th	6th	6th	$2.33x10^7$	$1.87x10^7$	$2.45x10^7$	$2.66x10^7$
C&GPrm (0% Maltodextrin)	-	-	-	-	$1.88x10^7$	$1.96x10^7$	$1.55x10^7$	$1.66x10^7$
C&GPrm-Md (1% Maltodextrin)	5th	5th	4th	4th	$1.57x10^7$	$1.44x10^7$	$1.91x10^7$	$1.81x10^7$
Basal Medium (1% Maltodextrin)	4th	-	5th	-	$2.01x10^7$	$2.11x10^7$	$1.79x10^7$	$1.90x10^7$
Basal Medium (0% Maltodextrin)	-	-	-	-	$6.5x10^2$	$5.8x10^2$	$5.1x10^2$	$5.3x10^2$

† The RPLA reaction profile, where the lowest dilution well where a positive latex agglutination reaction occurred is illustrated . The minus symbol designates a negative RPLA reaction.
‡ Where the test medium was initially inhabited by a 9 hour B. licheniformis cell culture prior to the addition of the test organism.
[a] The abbreviated test culture media ate explained in the above text.
[b,c] Represents the latex agglutination reaction for the filtrate and concentrate respectively.

Enterotoxin was not detected in the concentrate obtained from the *B. cereus* test culture which had been cultivated in a basal media supplemented with 1% maltodextrin, indicating that this ultracentrifugation successfully separated the toxin from the concentrate. However, this separation system did not successfully remove all the enterotoxin from the IMF (containing maltodextrin) concentrate samples.

Test media (either IMF or Basal Synthetic Media in origin) that did not contain maltodextrin failed to support the formation of diarrhoeal toxin. The cultivation of *B. licheniformis* prior to *B. cereus* in the same test medium did not appear to significantly enhance the level of toxin formation (P0.05).

The *B. licheniformis* 19 hour test culture was shown to be negative for enterotoxin formation, as was the *B. sphaericus* negative toxin control and the basal media. A similar RPLA reaction profile was achieved for *B. cereus* (NCTC 11145) cultivated in the complimentary set of test media (Table 5.21). This study revealed that the Intercep® Membrane Filtration System was a suitable technique for the separating the fat and diarrhoeal enterotoxin (produced by enterotoxigenic strains of *B. cereus*) present in contaminated infant milk formula.

Table 5.21 Detection of diarrhoeal enterotoxin in the filtrate and/or concentrate of a variety of test media previously cultured with *B. cereus II* (No. 42) via the BCET-RPLA test system.

Test medium	BCET-RPLA reaction†				Total Aerobic Mesophilic Count (CFU ml^{-1})			
	With‡ B. licheniformis		Without B. licheniformis		With B. licheniformis		Without B.licheniformis	
	Filtrate[b]	Concentrate[c]	Filtrate	Concentrate	Filtrate	Concentrate	Filtrate	Concentrate
FSM[a] (3.78% Maltodextrin)	7th	7th	6th	5th	$1.83x10^7$	$1.51x10^7$	$2.75x10^7$	$2.86x10^7$
C&GPrm (0% Maltodextrin)	-	-	-	-	$1.52x10^7$	$1.38x10^7$	$2.41x10^7$	$2.35x10^7$
C&GPrm-Md (1% Maltodextrin)	4th	4th	6th	3rd	$1.69x10^7$	$1.55x10^7$	$2.55x10^7$	$2.37x10^7$
Basal Medium (1% Maltodextrin)	5th	-	5th	-	$2.01x10^7$	$2.11x10^7$	$2.39x10^7$	$2.30x10^7$
Basal Medium (0% Maltodextrin)	-	-	-	-	$3.6x10^2$	$4.5x10^2$	$3.3x10^2$	$3.6x10^2$

† The RPLA reaction profile, where the lowest dilution well where a positive latex agglutination reaction occurred is illustrated. The minus symbol designates a negative RPLA reaction.
‡ Where the test medium was initially inhabited by a 9 hour B. licheniformis cell culture prior to the addition of the test organism.
[a] The abbreviated test culture media are explained in the above text.
[b,c] Represents the latex agglutination reaction for the filtrate and concentrate respectively.

5.4 The efficacy of infant bottle sanitation procedures at removing *Bacillus* cells/spores from reusable feed bottles and teats.

In most developed countries there is a high standard of hygiene in infant feeding, so that infantile gastro-enteritic disease is now rare (section 2.3.3). Due to either personal and/or medical reasons a mother may be unable to breast feed her baby, then a substitute feed in the form of reconstituted infant milk formulae is strongly recommended (section 2.3.1) In the interest of infant safety, it is of paramount importance that these milk based infant formulae remain germ free (or almost sterile) by applying suitable cleaning and sterilising (heating or chemical) treatments to the infant bottles and teats prior to actual formulae preparation. Indeed, failure to eliminate the microbiological build up (e.g. *B. cereus I or II*) could potentially result in an increased inoculum and enhanced contamination of a subsequently prepared batch of reconstituted infant formula. Results from section 5.3 revealed that the

storage of reconstituted infant milk formulae (IMF) under unfavourable storage conditions (i.e. established to be at temperatures in excess of 20°C for 14 hours or more) brought about an increase in the type, number and concentration of micro-organisms present. Furthermore, improper storage of these reconstituted milk based powders often amplified the concentration of indigenous spore-forming bacteria present to hazardous levels,i.e. levels at which these IMF fail internationally recognised safety or quality standards as laid down by all the governing bodies (section 2.3.2).

This study focused on the efficacy of commonly used cleaning (i.e. rinsing, washing and/or brushing) and sterilising (i.e. sodium hypochlorite, steam sterilisation, microwave steam sterilisation) procedures for removal and/ or elimination of the indigenous bacterial flora which could potentially occur in inadequately stored IMF samples (section 3.2.7). *Bacillus* spp. were cultivated in sterilised feeding bottles containing reconstituted IMF to population densities within a range found to be possible under normal domestic storage conditions (section 5.3). Moreover, the study employed two microbial sources, namely; the indigenous microbiological flora naturally present in unsterilised IMF and the aseptic inoculation of a pure strain of *Bacillus cereus II* into sterilised IMF. These naturally and artificially contaminated IMF were stored at 0 , 18 and 24 hours at the incubation temperatures of 4°C and 30°C. The extent to which *B. cereus* proliferated in these contaminated feeding bottles was monitored by carrying out a Total Aerobic Mesophilic Count (CFU ml^{-1}) on the TSYEA and BCSA plates. These defined temperature/time profiles permitted controlled microbial growth to known concentrations (CFU g^{-1} IMF). The efficacy of current cleaning and/or sterilising procedures (section 3.2.7) could then be assessed by monitoring the elimination and/or reduction in these pre-determined cell/spore concentrations (section 5.3).

The study then simulated the range of "after use" situations in the home and/or hospital, where the contents of the contaminated bottles were decanted and subjected to certain storage and/or cleaning regimes prior to sterilisation: a) the decanted unwashed bottles containing visible milk deposits were stored at the above temperatures for up to 24 hours; b) the emptied bottles were rinsed out and washed

prior to storage at the above temperatures for up to 24 hours; c) the bottles were rinsed and/or washed with sterile cold, warm, hot and boiling water with the use a bottle brush and/or household detergent removing all traces of milk deposits prior to storage at the above temperatures for up to 24 hours.

The efficacy of the cleaning procedures was then examined by aseptically adding sterile BHI broth (equal in volume to the IMF decanted) to the decanted cleaned bottles, followed by carrying out a Total Aerobic Mesophilic Count (CFU ml^{-1}) on duplicate bottle wash samples as described above.

The effectiveness of normal bottle 'sterilisation' treatments, such as the Boots® Complete Baby Feedtime Steriliser (i.e. chemical sterilising technique) and the Boots® Feedtime Steam Steriliser and Boots® Microwave Feeding Bottle Steam Steriliser (i.e. heat sterilising techniques) were assessed (section 3.2.7).

In addition to the generation of known cell concentrations, these established storage conditions could potentially permit the development of a microbial matrix or *biofilm* on the infant bottle and/or teat surfaces, making it more difficult to satisfactorily clean and sterilise the feeding apparatus.

Prior to reconstitution and storage of infant formulae, each bottle was thoroughly inspected for any physical scratches of cracks. New infant bottles (including dome cap, locking ring and sealing disc) and teat were thoroughly cleaned by brushing and rinsing in warm water which contained a detergent. The cleaned feeding bottles were then sterilised via Boots Feedtime Steam Steriliser (section 3.2.7) prior to use.

5.4.1 Efficacy of cleaning and/or steam sterilisation (via Boots® Feedtime Steam Steriliser) on the removal or elimination of *B. cereus II* from contaminated feeding bottles.

The effectiveness of different levels of cleaning were evaluated by initially inoculating 1 ml of an overnight culture of *B. cereus II* (NCTC 11145) into duplicate sets of 10 infant feeding bottles containing reconstituted SMA Gold Cap (IMF). The *B. cereus II* strain was cultivated in Brain Heart Infusion broth (BHI) at 125 rpm for 24 hours at

30°C prior to inoculation. These seeded infant formulae (where 30 g infant powder was reconstituted in 220 mls of sterile water at a water temperature of 56°C as described in section 5.1) were then incubated for 18 hours at either 4°C and/or 30°C as described in section 5.3. These temperature and time regimes provided suitable conditions for a defined level of microbial growth to take place. In order to obtain a homogeneous culture of *B. cereus 11,* the infant milk formula was tyndallised prior to inoculation.

After this 18 hour storage period the infant feeding bottles were cleaned prior to steam sterilisation as follows:

a) the contents of the feeding bottles was decanted (with no further cleaning).

b) the contents of the feeding bottles was decanted, rinsed 3 times in warm tap water.

c) the contents of the feeding bottles was decanted, washed and brushed 3 times in warm tap water containing detergent and rinse 3 times in warm tap water. Care was taken to ensure that all visible milk deposits were removed from the wall surface. In addition to the washing, brushing and rinsing of teats, all milk residues were removed from the teats by rubbing in salt (residual salt deposits were removed by rinsing in water).

One set of bottles was then steam sterilised via the Boots Feedtime Steam Steriliser (Figure 3.1) and then 250 ml of sterile BHI broth was then added to each of the bottles which had been either cleaned or cleaned and sterilised. Determination of the degree of bottle cleaning was obtained by aseptically adding an equivalent volume of sterile BHI broth to the decanted infant bottles, as this volume of BHI broth served as a bottle wash to establish the concentration of microbes present (CFU ml^{-1} BHI broth bottle wash). A total aerobic mesophilic count (CFU ml^{-1} wash) was carried out on TSYEA and BCSA plates via the spiral plater technique (section 3.2.9). Each set of cleaned or cleaned/steam sterilised bottles were subsequently stored at 4°C and 30°C for 18 hours (at which point a Total aerobic mesophilic count was carried out as described earlier). The determination of any significant difference between cleaning and/or sterilising procedures was achieve by applying the non-parametric Mann-Whitney U-test (for independent groups) and the Wilcoxon signed rank test (for related samples).

The study was repeated using an unsterilised IMF sample previously shown to contain only *B. cereus* (SMA While Cap, section 5.3). This IMF was aseptically added to sterile water (without any additional heat treatment) as described in section 5.1.

Examination of the results obtained in Tables 5.22 and 5.23 revealed that this commercial steam sterilising method was efficient at either reducing heavily microbially populated IMF to a satisfactory safe level for human consumption (ICMSF 1978) or eliminating the micro-organisms present to a level where these heat damaged bacteria were not recoverable using the standard agar plating techniques.

Indeed, examination of the information presented in these tables revealed that the greater the level of cleaning, the larger the reduction in cell concentration. The application of these 3 cleaning stages resulted in a progressive reduction in *B. cereus* cell number, where the bacterial load (CFU ml^{-1} bottle wash) obtained at each cleaning stage was significantly different. While the normal rinsing procedure reduced the bacterial load by a factor of 10^1-10^2 CFU ml^{-1}, further washing and brushing in warm soapy water only reduced the initial cell concentration by a factor of 10^2-10^3 CFU ml^{-1} bottle wash. Therefore, even the most thorough cleaning procedure (c) did not eliminate *B. cereus* from these cleaned (unsterilised) bottles. Indeed, application of the best cleaning regime (i.e. which removed all visible evidence of milk deposits) often resulted in Total Aerobic Mesophilic Count of $>10^4$ CFU ml^{-1}, which is a level of microbiological quality deemed to be unsatisfactory by the ICMSF (1986).

Storage of the decanted feeding bottles (previously containing either tyndallised SMA Gold Cap or unsterilised SMA White Cap) at the refrigeration temperature of 4°C did not permit any significant increase in microbial concentration. Therefore, the Total Aerobic Mesophilic Count (CFU g^{-1}) obtained after each cleaning regime (O hours storage) did not significantly change after an additional 18 hours storage at 4°C. These Total Aerobic Mesophilic Counts were not illustrated in this study as they were similar to mean counts obtained for the cleaned or partly cleaned feeding bottles analysed at 30°C (0 hours) in Tables 5.22 and 5.23.

Table 5.22 Efficacy of cleaning and/or steam sterilisation on the removal or elimination of *B. cereus II* from feeding bottles containing tyndallised SMA Gold Cap (IMF).

Treatment of reconstituted Infant feeding bottles‡	**Total Aerobic Mesophilic Count (CFU ml^{-1}) on BCSA plates†.**	
	0 hours storage at 30°C	**18 hours storage at 30°C**
Contents of bottles simply decanted.	$3.75x10^5$ Range ($4.0x10^5$-$5.9x10^6$)	$1.55x10^8$ Range ($1.0x10^7$-$1.5x10^8$)
Contents of bottles decanted and steamed for 15 mims (@100°C).	ND at 10^1 Range (ND at 10^1)	ND at 10^1 Range (ND 10^1)
Bottle decanted and rinsed 3 times in warm water.	$5.8x10^4$ Range ($1.9x10^4$-$4.1x10^4$)	$1.25x10^8$ Range ($8.6x10^7$ - $3.0x10^8$)
Bottles rinsed 3 times in warm water and steamed for 15 mins.	ND at 10^1 Range (ND at 10^1)	ND at 10^1 Range (ND at 10^1)
Bottles rinsed, washed and brushed 3 times in warm water containing detergent.	$4.85x10^5$ Range ($4.6x10^5$-$5.1x10^5$)	$7.0x10^7$ Range ($5.0x10^7$-$8.3x10^7$)
Bottles rinsed, washed and brushed 3 times in warm water containing detergent and then steamed for 15 mins.	ND at 10^1 Range (ND at 10^1)	ND (10^1) Range (ND at 10^1)
Sterile control feed (tyndallized) in sterile feeding bottles.	ND at 10^1 Range (ND at 10^1)	ND at 10^1 Range (ND at 10^1)

‡ Rinsing, washing, brushing and/or steaming treatment of infant feeding bottles post cultivation of a 24 hour culture of *B. cereus II* in 250 ml of SMA Gold Cap.
† 250 ml of sterile Brain Heart Infusion Broth (BHI) aseptically added to treated feeding bottles.

While thorough cleaning did not eliminate *B. cereus* from the feeding bottles, steam sterilisation successfully reduced the indigenous population to a non detectable level at 0 hours storage (Tables 5.22 and 5.23). However, additional storage of the partly cleaned bottles (previously containing SMA White Cap) over a 18 hour period resulted in the reappearance of *B. cereus* (Table 5.23). Thorough cleaning (procedure c), in conjunction with the application of Boots Feedtime Steam Sterilising technique resulted in the feeding bottles remaining free of this organism. Therefore, improper

cleaning of contaminated feeding bottles subsequent to steam sterilisation, may result in the survival and re-emergence of potentially pathogenic *Bacillus* spp. in inadequately stored bottles containing fresh formulae.

There was no significant difference (P0.005) between the reduction level of *B. cereus* in artificially (tyndallised SMA Gold Cap) and naturally (SMA White Cap) contaminated feeding bottles on cleaning.

Table 5.23 Efficacy of cleaning and/or steam sterilisation on the removal or elimination of *B. cereus II* from feeding bottles containing untyndallised SMA White Cap (IMF).

Treatment of reconstituted Infant feeding bottles‡	**Total Aerobic Mesophilic Count (CFU ml^{-1}) on BCSA plates†.**	
	0 hours storage at 30°C	**18 hours storage at 30°C**
Contents of bottles simply decanted.	$7.6x10^6$ Range ($6.8x10^6$-$8.4x10^6$)	$1.4x10^8$ Range ($7.6x10^7$-$2.33x10^8$)
Contents of bottles decanted and steamed for 15 mins (@100°C).	$1.8x10^3$ Range ($1.1x10^3$-$2.1x10^3$)	$8.6x10^7$ Range ($4.8x10^7$-$1.3x10^8$)
Bottle decanted and rinsed 3 times in warm water.	$2.6x10^5$ Range ($1.9x10^5$-$3.1x10^5$)	$2.1x10^8$ Range ($6.8x10^7$-$3.56x10^8$)
Bottles rinsed 3 times in warm water and steamed for 15 mins.	ND at 10^1 Range (ND at 10^1)	$4.5x10^5$ Range ($3.3x10^4$-$5.5x10^5$)
Bottles rinsed, washed and brushed 3 times in warm water containing detergent.	$2.3x10^4$ Range ($2.7x10^4$-$2.9x10^4$)	$9.1x10^7$ Range ($6.0x10^7$-$1.0x10^8$)
Bottles rinsed, washed and brushed 3 times in warm water containing detergent and then steamed for 15 mins.	ND at 10^1 Range (ND at 10^1)	ND (10^1) Range (ND at 10^1)
Sterile control feed (tyndallized) in sterile feeding bottles.	ND at 10^1 Range (ND at 10^1)	ND at 10^1 Range (ND at 10^1)

‡ Rinsing, washing, brushing and/or steaming treatment of infant feeding bottles subsequent to incubation of untyndallised SMA White Cap.

† 250 ml of sterile Brain Heart Infusion Broth (BHI) aseptically added to treated feeding bottles.

5.4.2 Efficacy of cleaning and/or steam sterilisation (via Boots® Microwave Feeding Bottle Steam Steriliser) on the removal or elimination of *B. cereus II* from contaminated feeding bottles.

In order to confirm the results obtained in section 5.4.1, the study was repeated using a different steam sterilising technique, i.e. the Boots® Microwave Feeding Bottle Steam Steriliser (Figure 3.2). While the same cleaning procedure was followed to that described in the previous study (section 5.4.1), the cleaned or partly cleaned feeding bottles were steam sterilised over a 8 minute period in a microwave oven as described in section 3.2.7. As there appeared to be no significant difference between the level of *B. cereus* reduced in cleaned or partly cleaned feeding bottles which contained either artificially (SMA Gold Cap) or naturally (SMA White Cap) contaminated IMF (section 5.4.1), the study was repeated using naturally contaminated infant feeding bottles as described in the previous study (section 5.4.1). The results of the cleaning and/or sterilising procedures were similar to those described in section 5.4.1. (Table 5.24). Examination of the information presented in this table revealed that cleaning alone was insufficient to reduce the bacterial load to a non-detectable level. However, thorough cleaning in addition to the microwave steam sterilising technique successfully eliminated *B. cereus II* from the contaminated feeding bottles at 0, 1 and 5 hours storage at 30°C. Unlike the previous study where *B. cereus II* was eliminated from contaminated feeding bottles after 18 hours storage (Table 5.22), thorough cleaning followed by a microwave steam sterilising treatment did not keep the infant bottles free of this organism. Indeed, examination of table revealed that, the less thorough the cleaning regime, the earlier the re-emergence of *B. cereus II*.

Table 5.24 Efficacy of cleaning and/or microwave steam sterilisation on the removal or elimination of *B. cereus II* from feeding bottles containing untyndallised SMA Gold Cap (IMF).

Treatment of reconstituted Infant Feeding Bottles[*].	**Total Aerobic Mesophilic Count (CFU ml^{-1}) on BCSA plates[†]**			
	0 hr at 30°C	**1 hr at 30°C**	**5 hr at 30°C**	**18 hr at 30°C**
Decant contents of bottles only (No further cleaning/sterilizing treatment carried out)	$3.4x10^6$ Range ($1.14x10^6$-$4.3x10^6$)	$2.6x10^6$ Range ($1.13x10^6$-$3.5x10^6$)	$6.8x10^6$ Range ($5.9x10^6$-$8.0x10^6$)	$2.9x10^8$ Range ($9.1x10^7$-$3.3x10^8$)
Decant contents of bottles followed by immediate steaming for 8 mins.	ND at 10^1 Range (ND at 10^1)	ND at 10^1 Range (ND at 10^1)	$3.0x10^3$ Range ($6.0x10^2$-$5.5x10^3$)	$1.56x10^8$ Range ($1.03x10^8$-$2.15x10^8$)
Decant, and rinse 3 times in warm water.	$2.3x10^4$ Range ($8.2x10^3$-$3.9x10^4$)	$1.95x10^4$ Range ($7.8x10^3$-$3.3x10^4$)	$2.8x10^6$ Range ($1.6x10^6$-$3.7x10^6$)	$1.29x10^8$ Range ($9.0x10^7$ $2.2x10^8$)
Decant, rinse 3 times in warm water and steam for 8 mins.	ND at 10^1	ND at 10^1	$4.0x10^2$ Range ($2.0x10^2$-$6.0x10^2$)	$8.1x10^7$ Range ($6.9x10^7$-$8.8x10^7$)
Decant, rinse 3 times in warm water, brush and wash in water & detergent.	$2.0x10^3$ Range (ND at 10^1- $5.0x10^3$)	$1.0x10^3$ Range (ND at 10^1- $3.0x10^3$)	$4.5x10^4$ Range ($5.0x10^3$)-$9.0x10^4$)	$1.56x10^8$ Range ($8.8x10^7$-$2.23x10^8$)
Decant, rinse 3 times in warm water, brush and wash in water & detergent and steam for 8 mins.	ND (10^1)	ND (10^1)	ND (10^1)	$5.0x10^4$ Range (ND at 10^1 - $7.0x10^4$
Sterile control feed (tyndallized) in sterile feeding bottles.	ND at 10^1	ND at 10^1	ND at 10^1	ND at 10^1

[*] Decanting and/or rinsing, washing, brushing steaming treatment of infant feeding bottles which had been initially cultivated by a 24 hour *B. cereus II* culture in 250 ml of Farlevs Oster Milk Two.
[†] Total aerobic mesophilic count is ml^{-1} BHI broth bottle wash, which is aseptically added (250 ml) to the treated feeding bottles and enumerated on BCSA plates.

5.4.3 Efficacy of cleaning and/or chemical sterilisation (via Boots® Complete Baby Feedtime Steriliser) on the elimination of *B. cereus II* from contaminated feeding bottles.

The aim of this study was to determine the effectiveness of chemical sterilisation on the elimination of *B. cereus II* from cleaned and partly cleaned infant feeding bottles.

Sterilised infant feeding bottles containing SMA White Cap (i.e. previously shown to contain only *B. cereus II*) was stored for 24 hours at 30 prior to cleaning and sterilising (section 5.4.2).

The duplicate series of contaminated feeding bottles were cleaned as described in the previous section 5.4.3 and then chemically sterilised using the Boots® Complete Baby Feedtime Steriliser (section 3.2.7) as described in Table 5.25. An equivalent volume of 250 ml BHI broth was aseptically added to the decanted and/or cleaned bottles. A Total Aerobic Mesophilic Count (CFU ml^{-1} BHI bottle wash) was carried out on the bottle wash after 0 and 18 hours incubation at 30°C as described in section 5.4.1.

The results of this cleaning and/or chemical sterilising treatment are shown in Table 5.25. Examination of this table revealed that chemical sterilisation successfully removes a high bacteriological load if the contaminated feeding bottles are totally immersed in 125 ppm Milton Solution for the recommended 30 mins (Figure 3.3).

Table 5.25 Efficacy of cleaning and/or chemical sterilisation (via Boots® Complete Baby Feedtime Steriliser) on the elimination of *B. cereus II* from contaminated feeding bottles.

Treatment of reconstituted of Infant Feeding Bottles‡	**Total Aerobic Mesophilic Count (CFU ml^{-1}BHI broth wash)†**	
	0 hour at 30°C	**18 hour at 30°C**
Decanted contents of infant Bottles only.	$1.21x10^7$ Range ($9.3x10^6$ - $1.75x10^7$)	$1.5x10^8$ Range (1.16 - $1.81x10^8$)
Decanted contents of feeding bottles, and rinsed out immediately with 125 ppm Milton Solution.	$5.56x10^5$ Range ($4.8x10^5$ - $6.1x10^5$)	$1.25x10^8$ Range ($9.6x10^7$ - $1.44x10^8$)
Decanted contents, rinsed,washed,and brushed in warm water & detergent, and totally immersed in 125 ppm Milton Solution for 30 mins.	ND at 10^1	$8.5x10^5$ Range ($4.2x10^5$ - $2.2x10^6$)
Decant contents of bottle and immersed directly in 125 ppm Milton Solution where it was stored for 30 mins.	ND at 10^1	$3.8x10^6$ Range (3.35 - $4.1x10^6$)
Decanted contents of sterile IMF (non-inoculated) control.	ND at 10^1	ND at 10^1

‡ Degree of cleaning of contaminated feeding bottles which initially contained SMA Gold Cap (an IMF which was previously stored overnight and contained *B. cereus*).

† Total aerobic mesophilic count (CFU ml^{-1} BHI wash) is per ml BHI broth bottle wash,where 250 mls of BHI broth was aseptically added to the treated feeding bottles. Surviving *B. cereus II* propagules were enumerated on Bacillus Cereus Selective Agar (BCSA).

However, additional storage of these chemically sterilised bottles containing a suitable growth medium (e.g. BHI broth), allowed the recovery of *B. cereus* cells after 18 hours. A brief exposure of the contaminated feeding bottles to 125 ppm Milton solution did not eliminate *B. cereus*. The results of these bottle cleaning and sterilising experiments (section 5.4.1 to 5.4.3) revealed that thorough cleaning followed by either a chemical or heat sterilising treatment was sufficient to eliminate *B. cereus* from feeding bottles (where *B. cereus* was present at a 10^7 CFU ml^{-1}).
This and the other studies also revealed that properly cleaned and sterilised feeding bottles containing fresh infant formulae should not be allowed to sit for prolonged periods at an ambient temperature, as either *Bacillus spp.* attached to the inner bottle or teat surfaces (from an earlier feed) or indigenous flora in the fresh feed may grow to dangerously high levels (>10^4 CFU ml^{-1}).

5.5 A case study on the microbial quality of reconstituted infant formulae prepared in a special feeding unit at the Royal Hospital for Sick Children, Yorkhill, Glasgow.

The objective of the study was to determine the number, type and concentration (Total Aerobic Mesophilic Count expressed as Colony Forming Units ml^{-1} IMF) of *Bacillus spp.* and/or *Listeria spp.* present in reconstituted infant formulae stored over a 24 hour period at a refrigerated temperature of 4°C and at an ambient room temperature of 25°C. The microbial growth profiles of each of these bacteria were monitored over the duration of the storage periods. Diarrhoegenic enterotoxin production was assayed for in all infant formulae which had been previously shown to be colonialised by *B. cereus II.*
A total of 25 infant formulae were collected on 3 separate visits to the Special Feeding Unit at the Royal Hospital For Sick Children (Yorkhill, Glasgow). The infant formulae supplied for microbiological analysis was a representative sample of the actual IMF supplied to the hospital wards and was therefore tailor made for the specific requirements of the individual patients (i.e. the standard infant formulae were

often supplemented with hydrolysed protein, feed thickeners, glucose polymers, fat calorie supplements *etc.*). Twenty five mls of each type of infant formulae was transferred into a separate sterile universal bottle and labelled with the date, time of preparation, description of product and any other comment post reconstitution and pasteurisation at Special Feeding Unit one hour prior to collection.

The samples were then transported to the laboratory in a cooler bag containing two ice packs and a maximum/minimum thermometer which were included to regulate the storage temperature on transit. It took on average 40 mins to transport the samples to the laboratory and the temperature range of the cooler bag throughout transit was -1°C ± 3°C. The package integrity of each plastic universal containers was checked (e.g. cracks in wall of container) prior to bacteriological analysis.

Each infant formulae sample was spirally plated in triplicate onto Nutrient Agar containing 0.6% manganese sulphate (NAMS), *Bacillus cereus Selective Agar* (BCSA), and 7% defribrinated horse Blood Agar (BA) after 0 hr, 8 hr, 14 hr and 24 hr storage at 4°C and/or 25°C (section 5.1). A 5 ml sample was analysed in duplicate for the presence of *Listeria spp.* (particularly *L. monocytogenes*) as described in section 5.1. All these inoculated agar plates were then incubated aerobically at 30°C and/or 37°C for 24 hr and 48 hr. Diarrhoeal enterotoxin production by certain strains of *B. cereus II* was confirmed in both the IMF and a artificial laboratory media (i.e. Brain Heart Infusion Broth supplemented with 0.25% glucose) via the Reverse Phase Latex Agglutination immunological test kit (section 3.9).

The microbiological quality of these reconstituted IMF samples (supplied by Yorkhill Hospital, Glasgow) was similar to that exhibited by the 100 IMF samples analysed in section 5.3. According to the specifications laid down by the International Commission on Microbiological Specifications for Food (1986), 23 (out of a total of 25) samples analysed had a satisfactory Total Aerobic Count and *B. cereus* Count of not more than $1.0x10^4$ and 10^3 CFU ml^{-1} respectively, one sample (Table 5.29) exhibited a *B. cereus I* count greater than the recommended $1.0x10^3$ CFU g^{-1} (Tables 5.26, 5.27, 5.28 and 5.29), and one was not analysed due to a failure in its package integrity. *Listeria spp.* were not recovered from any of the samples over the course of the investigation.

The total aerobic mesophilic counts represented in these tables (5.26 to 5.29) are expressed in CFU ml^{-1} IMF as the infant formulae was supplied in a reconstituted form. In order to relate these individual total viable counts to the ICMSF standards (1978) (which is expressed in g^{-1} powder) it was necessary to adjust these counts by a factor of 10, in order to allow for the 1 in 10 dilution of the infant powder on reconstitution. However, of the *Bacillus spp.* isolated from infant formulae samples during this study: *B. cereus I and II*, *B. licheniformis*, *B. subtilis*, *B. pumilus*, *B. circulans*, *B. sphaericus*, *B. brevis*, *B. megaterium* and *B. mycoides* have been implicated in either food related illnesses and/or opportunist infections (section 2.3.2). Examination of the information presented in these tables revealed a variation in the type, number and concentration (expressed as colony forming units ml^{-1} IMF analysed) of *Bacillus spp.* recovered from different infant formulae (IF) when stored over a 24 hr period at 25°C. Moreover, storage of samples for 14 hr or more at an incubation temperature of 25°C resulted in a general increase in number, type and concentration (CFU ml^{-1}) of *Bacillus spp*, while storage for shorter duration's (i.e. $\leq$ 8 hr) and/or at the lower refrigeration temperature of 4°C did not significantly enhance the microbial number (P 0.05 Wilcoxin rank sign test).

The number and type of *Bacillus spp.* recovered over the 24 hour period at both refrigeration and ambient room temperatures are shown in Table 5.30. At the early stages of storage, members of the *B. subtilis group* (namely *B. licheniformis* and *B. subtilis*) were the most frequently isolated aerobic spore-formers recovered from infant formulae, while additional storage ($\geq$14 hours) brought about a significant increase in the number, type and concentration (CFU ml^{-1} IMF) of *Bacillus spp.* present (with the emergence of *B. cereus* as dominant organisms).

Diarrhoeal enterotoxin production was confirmed by 3 of the 5 *B. cereus II* strains when they were cultivated in infant formulae and in the artificial laboratory medium (BHI supplemented with glucose) at 30°C for 20 hours under orbital incubation at 110 rpm.

Table 5.26 Variation in number, type and concentration (CFU ml^{-1} IMF) of *Bacillus spp.* obtained from infant milk formulae on storage over a 24 hour period at 4°C and 25°C.

STORAGE CONDITIONS (°C)	BACILLUS SPECIES	TOTAL AEROBIC COUNT (CFU/ ml^{-1} IMF)							
		0 Hours		4 Hours		14 Hours		20 Hours	
		4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°
SMA Gold Cap	*B. licheniformis*	$2.0x10^1$	$2.0x10^1$	$4.0x10^1$	$1.0x10^1$	$1.0x10^1$	$6.0x10^1$	$1.0x10^1$	$6.5x10^2$
	B. sphaericus	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	$4.0x10^1$
	Other Bacillus spp	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$2.0x10^1$**	**$2.0x10^1$**	**$4.0x10^1$**	**$1.0x10^1$**	**$1.0x10^1$**	**$6.0x10^1$**	**$1.0x10^1$**	**$6.9x10^2$**
SMA White Cap	*B.licheniformis*	$4.0x10^1$	$4.0x10^1$	$5.0x10^1$	$4.0x10^1$	$4.0x10^1$	$8.0x10^1$	$2.0x10^1$	$9.4x10^2$
+ Duocal	*B. sphaericus*	ND(10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	ND(10^1)	$3.0x10^1$	ND (10^1)	$2.5x10^2$
+ Carobel	*Other Bacillus spp*	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$4.0x10^1$**	**$4.0x10^1$**	**$5.0x10^1$**	**$5.0x10^1$**	**$4.0x10^1$**	**$1.2x10^2$**	**$2.0x10^1$**	**$1.19x10^3$**
Premcare Formula	*B. licheniformis*	$5.0x10^1$	$3.0x10^1$	$3.0x10^1$	$5.0x10^1$	$4.0x10^1$	$1.2x10^2$	$2.0x10^1$	$1.6x10^3$
+ ½ Strength Carobel	*B. cereus 2**	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	$5.0x10^1$	ND (10^1)	$1.3x10^3$
+ Duocal	*B. pumilus*	ND 10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	$1.0x10^1$	ND (10^1)	$2.0x10^2$
	Other Bacillus spp	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (101)	ND (10^1)
	Total Bacillus sp	**$5.0x10^1$**	**$3.0x10^1$**	**$3.0x10^1$**	**$5.0x10^1$**	**$4.0x10^1$**	**$1.8x10^2$**	**$2.0x10^1$**	**$1.3x10^3$**
Generaid Plus	*B. cereus 1*	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	$4.0x10^1$	ND (10^1)	$8.3x10^4$
	B. mycoides	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	$2.0x10^1$	ND (10^1)	$5.0x10^2$
	Other Bacillus spp	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**ND(10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND(10^1)**	**$6.0x10^1$**	**ND (10^1)**	**$1.33x10^3$**
Nutrilon Plus	*B. licheniformis*	$2.0x10^1$	ND (10^1)	ND (10^1)	$1.0x10^1$	ND(10^1)	$3.0x10^1$	$1.0x10^1$	$8.7x10^2$
+ Maxijul	*B subtilis*	ND 10^1)	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	ND(10^1)	$4.0x10^1$	ND (10^1)	$1.1x10^3$
	Other Bacillus spp	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$2.0x10^1$**	**$1.0x10^1$**	**$1.0x10^1$**	**$2.0x10^1$**	**ND(10^1)**	**$7.0x10^1$**	**$1.0x10^1$**	**$1.97x10^3$**
Monogen	*B. licheniformis*	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	$2.0x10^1$
	B. cereus 1	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	$3.2x10^2$
	B. megaterium	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	$5.0x10^1$
	Other Bacillus spp	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**ND(10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND(10^1)**	**ND (10^1)**	**ND (10^1)**	**$3.9x10^2$**
Nutrilon Plus	*B. subtilis*	$2.0x10^1$	$2.0x10^1$	$3.0x10^1$	$2.0x10^1$	$1.0x10^1$	$6.0x10^1$	$2.0x10^1$	$1.3x10^3$
+ Carobel	*Other Bacillus spp*	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$2.0x10^1$**	**$2.0x10^1$**	**$3.0x10^1$**	**$2.0x10^1$**	**$1.0x10^1$**	**$6.0x10^1$**	**$2.0x10^1$**	**$1.3x10^3$**

Table 5.27 Variation in number, type and concentration (CFU ml^{-1} IMF) of *Bacillus spp.* obtained from infant milk formulae on storage over a 24 hour period at 4°C and 25°C.

STORAGE CONDITIONS (°C)	BACILLUS SPECIES	TOTAL AEROBIC COUNT (CFU/ ml^{-1} IMF)							
		0 Hours		4 Hours		14 Hours		20 Hours	
		4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°
WYSOY	***Total Bacillus spp***	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND(10^1)**	**ND (10^1)**	**ND (10^1)**
Premcare Formuls + Duocal	*B. licheniformis*	$1.0x10^1$	$2.0x10^1$	$1.0x10^1$	ND (10^1)	$1.0x10^1$	$5.0x10^1$	$2.0x10^1$	$6.1x10^2$
	B. cereus 1	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$5.0x10^1$	ND (10^1)	$6.2x10^4$
	B. sphaericus	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)	$2.0x10^1$	ND (10^1)	$5.0x10^4$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$1.0x10^1$**	**$2.0x10^1$**	**$1.0x10^1$**	**ND (10^1)**	**$1.0x10^1$**	**$1.2x10^2$**	**$2.0x10^1$**	**$1.12x10^5$**
Nutramigen + Maxijul	*B. licheniformis*	$5.2x10^2$	$4.8x10^2$	$5.6x10^2$	$5.3x10^2$	$4.9x10^2$	$2.3x10^3$	$5.5x10^2$	$8.3x10^3$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND(10^1)	ND (10^1)
	Total Bacillus spp	**$5.2x10^2$**	**$4.8x10^2$**	**$5.6x10^2$**	**$5.3x10^2$**	**$4.9x10^2$**	**$2.3x10^3$**	**$5.5x10^2$**	**$8.3x10^5$**
Farleys Oster Milk Two	*B. subtilis*	$3.0x10^1$	$3.0x10^1$	$2.0x10^1$	$2.0x10^1$	$4.0x10^1$	$4.0x10^1$	$2.0x10^1$	$2.8x10^2$
	B. mycoides	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	ND (10^1)	ND (10^1)	$3.0x10^1$	ND (10^1)	$2.3x10^5$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$4.0x10^1$**	**$4.0x10^1$**	**$3.0x10^1$**	**$2.0x10^1$**	**$4.0x10^1$**	**$7.0x10^1$**	**$2.0x10^1$**	**$2.3x10^5$**
	B. sphaericus	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$4.0x10^1$	ND (10^1)	$8.1x10^3$
	B. mycoides	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	ND (10^1)	$2.4x10^3$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**$5.0x10^1$**	**ND (10^1)**	**$8.34x10^4$**
Nutrison Energy Plus	***Total Bacillus spp***	**ND (101)**	**ND (101)**	**ND (101)**	**ND (101)**	**ND (101)**	**ND (101)**	**ND (101)**	**ND (101)**
Elemental 028	*B. cereus 2**	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$2.0x10^1$	ND (10^1)	$3.5x10^4$
	B. megaterium	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	ND (10^1)	$2.3x10^4$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**ND (10^1)**	**ND (10^1)**	**Nd (10^1)**	**ND (10^1)**	**ND (10^1)**	**$3.0x10^1$**	**ND (10^1)**	**$5.8x10^4$**
Paedriatric Nutrison	***Total Bacillus spp***	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**	**ND (10^1)**
SMA White Cap	*B. licheniformis*	$1.0x10^1$	$1.0x10^1$	$2.0x10^1$	$1.0x10^1$	$1.0x10^1$	$6.0x10^1$	$1.0x10^1$	$2.4x10^4$
	Other Bacillus spp	ND (101)	ND (101)	ND (101)	ND (101)	ND (101)	ND (1010	ND (101)	ND (101)
	Total Bacillus spp	**$1.0x10^1$**	**$1.0x10^1$**	**$2.0x10^1$**	**$1.0x10^1$**	**$1.0x10^1$**	**$6.0x10^1$**	**$1.0x10^1$**	**$2.4x10^4$**

Table 5.28 Variation in number, type and concentration (CFU ml^{-1} IMF) of *Bacillus spp*. obtained from infant milk formulae on storage over a 24 hour period at 4°C and 25°C.

STORAGE CONDITIONS (°C)	*BACILLUS SPECIES*	TOTAL AEROBIC COUNT (CFU/ ml^{-1} IMF)							
		0 Hours		4 Hours		14 Hours		20 Hours	
		4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°
SMA Gold Cap	*B. subtilis*	$1.0x10^1$	$1.0x10^1$	$2.0x10^1$	$2.0x10^1$	ND (10^1)	$2.0x10^1$	ND (10^1)	$7.1x10^2$
	B. pumilus	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	$2.0x10^1$	ND (10^1)	$1.9x10^3$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$2.0x10^1$**	**$2.0x10^1$**	**$3.0x10^1$**	**$3.0x10^1$**	**$1.0x10^1$**	**$4.0x10^1$**	**ND (10^1)**	**$2.6x10^2$**
Pregestumil	*B. cereus 1*	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$3.0x10^1$	ND (10^1)	$2.1x10^3$
	B. polymyxa	$1.0x10^1$	$1.0x10^1$	ND (10^1)	$2.0x10^1$	$2.0x10^1$	$4.0x10^1$	$1.0x10^1$	$5.0x10^4$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
WYSOY	*B. licheniformis*	$3.0x10^1$	$2.0x10^1$	$1.0x10^1$	$1.0x10^1$	$1.0x10^1$	$6.0x10^1$	ND (10^1)	$2.4x10^3$
	B. sphaericus	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	ND (10^1)	$1.3x10^5$
	B. circulans 2	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	ND (10^1)	$4.1x10^4$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$3.0x10^1$**	**$2.0x10^1$**	**$1.0x10^1$**	**$1.0x10^1$**	**$1.0x10^1$**	**$8.0x10^1$**	**ND (10^1)**	**$1.73x10^5$**
Monogen	*B. licheniformis*	$3.0x10^1$	$3.0x10^1$	$5.0x10^1$	$3.0x10^1$	$4.0x10^1$	$1.1x10^2$	$3.0x10^1$	$5.0x10^2$
	B. cereus 1	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$3.0x10^1$	ND (10^1)	$1.5x10^5$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$3.0x10^1$**	**$3.0x10^1$**	**$5.0x10^1$**	**$3.0x10^1$**	**$4.0x10^1$**	**$1.4x10^2$**	**$3.0x10^1$**	**$1.5x10^5$**
Nutrilon Plus + Duocal	*B. cereus 1*	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$5.0x10^1$	ND (10^1)	$2.2x10^3$
	B. cereus 2	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$3.0x10^1$	ND (10^1)	$1.7x10^3$
	Cocci	$5.0x10^1$	$7.0x10^1$	$5.0x10^1$	$6.0x10^1$	$4.0x10^1$	$3.2x10^2$	$4.0x10^1$	$4.0x10^6$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$5.0x10^1$**	**$7.0x10^1$**	**$5.0x10^1$**	**$6.0x10^1$**	**$4.0x10^1$**	**$4.0x10^2$**	**$4.0x10^1$**	**$4.39x10^6$**
SMA White Cap + Maxijul	*B. licheniformis*	ND (10^1)	ND (10^1)	ND (10^1)	$1.0x10^1$	$1.0x10^1$	$5.0x10^1$	ND (10^1)	$2.1x10^3$
	B. megaterium	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$3.0x10^1$	ND (10^1)	$5.8x10^4$
	B. brevis	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$2.0x10^1$	ND (10^1)	$4.3x10^4$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	**$1.0x10^1$**	**$1.0x10^1$**	**$1.0x10^2$**	**ND (10^1)**	**$1.0x10^5$**

Table 5.29 Variation in number, type and concentration (CFU ml^{-1} IMF) of *Bacillus spp.* obtained from infant milk formulae on storage over a 24 hour period at 4°C and 25°C.

STORAGE CONDITIONS (°C)	BACILLUS SPECIES	TOTAL AEROBIC COUNT (CFU/ ml^{-1} IMF)							
		0 Hours		4 Hours		14 Hours		20 Hours	
		4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°
SMA Gold Cap	*B. licheniformis*	$1.0x10^1$	$1.0x10^1$	$2.0x10^1$	$3.0x10^1$	$3.0x10^1$	$1.0x10^1$	$1.0x10^1$	$9.0x10^2$
+ ½ Strength Nestragel	*B. subtilis*	$2.0x10^1$	$3.0x10^1$	$2.0x10^1$	$3.0x10^1$	$5.0x10^1$	$1.0x10^1$	$2.0x10^1$	$1.2x10^3$
+ Duocal	*B. cereus 2**	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	$2.0x10^1$	ND (10^1)	$1.1x10^5$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$3.0x10^1$**	**$4.0x10^1$**	**$4.0x10^1$**	**$6.0x10^1$**	**$1.0x10^2$**	**$2.0x10^4$**	**$3.0x10^1$**	**$1.11x10^5$**
SMA Gold Cap	*B. licheniformis*	$3.0x10^1$	$3.0x10^1$	$3.0x10^1$	$2.0x10^1$	$1.0x10^1$	$4.0x10^1$	$2.0x10^1$	$2.4x10^5$
	Other Bacillus spp	ND (10^1)	ND (10^1)	ND (10^1)	ND (101)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$3.0x10^1$**	**$3.0x10^1$**	**$3.0x10^1$**	**$2.0x10^1$**	**$1.0x10^1$**	**$4.0x10^1$**	**$2.0x10^1$**	**$2.4x10^5$**
Nutrilon Plus	*B. cereus 1*	$1.4x10^3$	$1.2x10^3$	$1.4x10^3$	$1.7x10^3$	$1.3x10^3$	$9.4x10^3$	$1.1x10^3$	$8.6x10^6$
+ ½ Strength Nestargel	*Other Bacillus spp*	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)	ND (10^1)
	Total Bacillus spp	**$1.4x10^3$**	**$1.2x10^3$**	**$1.4x10^3$**	**$1.7x10^3$**	**$1.3x10^3$**	**$9.4x10^3$**	**$1.1x10^3$**	**$8.6x10^6$**

* Represents diarrhoeal enterotoxin producing strains of *B. cereus ii*

Table 5.30 The microbiological quality of 24 IMF samples examined over a 24 hour period at a storage temperature of 4°C and 25°C.

Bacillus Spp. **Detected**	**The number of IMF samples supporting growth of a particular *Bacillus spp.* over a 24 hr period at 4°C and 25°C storage temperatures.¶**							
	0 hr		**8 hr**		**14 hr**		**24 hr**	
	4°C‡	**25°C†**	**4°C**	**25°C**	**4°C**	**25°C**	**4°C**	**25°C**
B. licheniformis	11	11	11	11	11	13	10	13
B. subtilis	5	5	5	5	4	5	4	5
B. pumilus	1	1	1	1	1	1	1	2
B. cererus I	1	1	1	1	1	6	1	6
B. cereus II	-	-	-	-	-	3	-	5 (3*)
B. megaterium	-	-	-	-	-	3	-	4
B. mycoides	1	1	1	1	1	3	1	3
B. polymyxa	1	1	-	1	1	1	1	1
B. circulans 2	-	-	-	-	-	1	-	1
B. brevis	-	-	-	-	-	1	-	1
B. sphaericus	-	-	-	-	-	5	-	6
Cocci	1	1	1	1	1	1	1	1
No Bacillus spp. detected	8	3	8	3	8	3	9	3
Listeria spp.	-	-	-	-	-	-	-	-

† IMF samples stored over a 24 hr period at 25°C.
‡ IMF samples stored over a 24 hr period at 4°C
* The number of *B. cereus II* strains demonstrating diarrhoegenic toxin production via RPLA system.
¶ The number of IMF samples supporting growth of a particular organism, where the total number of IMF sampled was 254and eiither BCSA , LSA, BA and/or TSYEA was the recovery medium employed.

6 Antagonistic culture studies

6.1 Antagonistic interaction between *Bacillus species* isolated from infant milk formulae and *Listeria monocytogenes*.

Increasing requirements to ensure a high quality, safe food supply combined with the continual threat of established and emerging opportunist microbial pathogens, has left the food industry searching for improved and more efficient procedures for the subsequent detection and control of these microorganisms in food products.

Exploitation of antagonism is generally based on the production of antimicrobial substances which injure or kill other undesirable microorganisms and as such, has been recognised as a natural process of food preservation. It is important to underline that such an antagonism is a biological process which has the potential of reducing the use of artificial products for the subsequent preservation of foods. It is has been shown that the indigenous microbial flora of reconstituted infant milk powder consists primarily of aerobic spore forming bacteria belonging to the *Bacillus* genus (section 2.3.2). If low levels of either heat damaged or non heat injured *L. monocytogenes* contaminants are to get established in reconstituted infant milk powders, they must first commonly share the same environment with these spore-forming microorganisms. While a number of studies have been carried to investigate the antagonistic interaction between asporogenic bacteria (e.g. members of the genus *Pediococcus*, *Lactobacillus*, *Streptococcus)* and *L. monocytogenes*, very little work is currently available regarding the possible competitive and/or non-competitive interactions between *Bacillus* (isolated from IMF) and *Listeria*.

Taking into account the considerations mentioned above, it was decided to carry out an in depth investigation into possible antagonistic interactions between members of the *Bacillus* species isolated from the 125 infant milk formula (section 5) and pleomorphic surface cultures of *L. monocytogenes* (section 4). An initial screening of the 178 *Bacillus* species (isolated from IMF) was carried out in order to presumptively identify possible antagonistic *Bacillus* isolates using an optimised

conventional antagonistic method described by Batista (1993). The author employed these antagonistic methods to successfully confirm an antagonistic interaction between *Bacillus amyloliquefaciens* and *L. monocytogenes*. Having determined the *Bacillus* isolates which exhibited possible antagonistic interactions with *L. monocytogenes*, a sequence of in depth direct and deferred antagonistic methods were carried out to confirm the degree and nature of this *Bacillus* induced antagonism. Additional studies were performed to establish whether the inhibitory process could be directed towards other microorganisms (including other members of the genus *Listeria)*.

Furthermore, direct antagonism is characterised by the simultaneous growth of a test organism (i.e. *Bacillus*) and an indicator organism (i.e. *Listeria*) on or in a culture medium, while deferred antagonism is characterised by the growth of the test (or indicator) organism on or in a culture medium prior to the addition of the indicator (or test) organism. Using both the direct and deferred antagonistic methods mentioned earlier, the ability of *Bacillus* isolates to inhibit the growth of smooth (NCTC 11994, NCTC 9863) and rough (NRB2) colony forms of *L. monocytogenes* were evaluated. These antagonistic interactions were observed in three culture media forms, namely: solid media (agar plates), semisolid media (soft agar) and liquid media (broth) at psychrotrophic and mesophilic temperatures. These antagonistic assays are described in detail below.

6.1.1 Antagonistic assay in solid media (agar plates)

A total of 178 *Bacillus* species (infant milk formulae isolates) were assessed for the ability to produce an antagonistic response against smooth and rough forms of *L. monocytogenes* in solid media using simultaneous and deferred methods (section 3.14). The former antagonistic method being employed first.

In order to confirm that initial antagonistic screening method (i.e. simultaneous technique as adopted by Batista 1993) provided optimum conditions to identify antagonistic interactions, the following key properties were subsequently assessed: type of assay media; inoculation level of *L. monocytogenes* (i.e. the indicator

organism), indicator cell inoculation technique; temperature of assay and type of test organism (i.e. *Bacillus* spp) employed. The purpose of these optimisation studies was to confirm that the earlier antagonistic screening procedure satisfactorily identified potential antagonistic *Bacillus* isolates (when challenged against *L. monocytogenes*). Three different types of culture media were employed: Tryptone Soya Yeast Extract Agar (TSYEA), Brain Heart Infusion Agar (BHIA) and Blood Agar Base, the latter medium being supplemented with 7% defribrinated horse blood (BA).

The initial antagonistic interactions were performed using different inoculum levels of *L. monocytogenes* (which ranged from 10^3 to 10^7 CFU ml^{-1}), in addition to two cultivation temperatures (35°C and 30°C). A detailed account of the initial screening studies aimed at identifying presumptive antagonistic *Bacillus* species are described below.

6.1.1.1 Simultaneous or direct antagonism

The ability of 178 *Bacillus* spp. (i.e. isolated from reconstituted infant milk formulae in section 5) to exhibit an antagonistic response against both smooth and rough colony forms of *L. monocytogenes* was evaluated via simultaneous and deferred antagonistic techniques (section 3.14). The *Bacillus* isolates were resuscitated from the freeze dried state and streaked to single colonies on TSYEA, allowing 24 hours at 35°C for the appearance of good growth. The indicator organism *(L. monocytogenes* NCTC 11994) was cultivated under gyrotary conditions at 110 rpm for 20 hours at 35°C in TSYEB to a final cell population level of approximately 10^9 cells ml^{-1} and tenfold dilutions (down to the 10^{-4} dilution) were performed. A 0.1 ml aliquot of this 20 hour 10^{-4} dilution was aseptically inoculated into a fresh set of TSYEB and incubated under the same set of conditions to ensure a homogeneous distribution of cells.

In order to standardise the *Listeria* cell number in each indicator test flask, the same procedure was repeated for *L. monocytogenes* NCTC 9863 and NRB2. A tenfold serial dilution (down to 10^{-3}) was carried out on each indicator culture and a 0.1 ml aliquot of this 10^{-3} dilution was spread across the surface of a TSYEA plate. A total

aerobic mesophilic count (CFU ml^{-1}) was carried out on TSYEA and LSA plates to confirm the indicator *(L. monocytogenes)* inoculation levels were approximately equal for the 3 strains.

Immediately after, the 178 *Bacillus* isolates were separately seeded by the conventional stab inoculation technique (section 3.14.2) and a maximum of five inoculations of the test organism (i.e. *Bacillus*), spaced 2 cm apart on duplicate plates were carried out. This direct stab antagonistic method was repeated for *L. monocytogenes* NCTC 9863 (a smooth colony form) and NRB2 (a rough colony form). The plates were then incubated at 35°C for 24 hours.

After incubation, the inhibitory activity was detected by the presence of a zone of clearing around the *Bacillus* colony. The level of antagonism demonstrated by the test organism (*Bacillus spp.*) against the indicator organism *(L. monocytogenes)* was established by simply measuring the zone of inhibition, as the distance from the outer colony margin or edge of the *Bacillus* isolate to the edge of the *Listeria* growth zone.

Results from the above study revealed that a diffuse inhibition zone of clearing was detected around 40 *Bacillus spp.* (22.5%) on the surface of the TSYEA plates via this stab inoculation technique. The results of this preliminary antagonistic screen are illustrated in Table 6.1, where the inhibition zone (mm) produced against each colony form of *L. monocytogenes* is the result of an average measurement made of the 5 stab inoculations on the duplicate plates taken from duplicate investigations (*i.e.* the mean of 20 inhibition zone measurements). The zones of inhibition produced as a result of antagonistic interaction between *Bacillus spp.* (i.e. stabbed test culture) and *L. monocytogenes* (seeded indicator culture) using the simultaneous surface technique on TSYEA plating media are shown in Figure 6.1.

Under the present set of antagonistic conditions, a variation in the size of the *Listeria* inhibitation zone was observed for different *Bacillus* isolates ranging from weak (0.5 mm) to very strong (5.0 mm) inhibition. This variation in *Listeria* growth inhibition was evident for both pleomorphic surface culture forms. Indeed the type of indicator colony form did not significantly affect the degree of antagonism exhibited by *L. monocytogenes* (at P0.05 level, Wilcoxon ranked Sign test). The mean TSYEA

surface inoculation level for *L. monocytogenes* NCTC 11994, NCTC 9863 and NRB2 was $4.2x10^5$, $4.6x10^5$ and $3.57x10^5$ CFU ml^{-1} respectively.

While an assay temperature of 35°C was satisfactory for the identification of definite diffuse zones of *Listeria* clearing produced by certain *Bacillus* species (e.g. *B. cereus*, *B. megaterium* and *B. mycoides)* on interaction with *L. monocytogenes*; it was not possible to successfully screen a large number of assay plates which had been stab inoculated with members of the *Bacillus subtilis* morphological grouping. These particular test organisms had overgrown or swarmed the assay plates making identification of *Listeria* inhibition zones impossible.

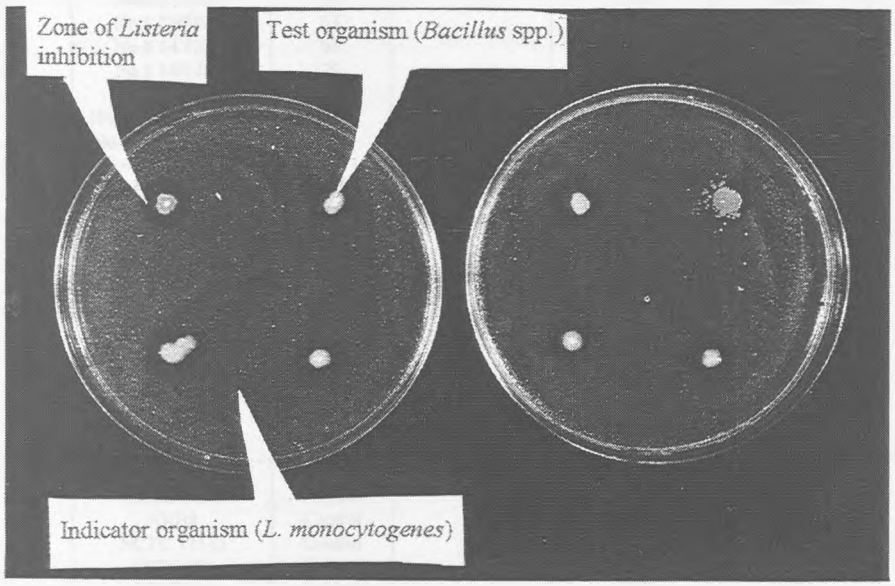


Figure 6.1 Direct antagonistic technique- zones of *Listeria monocytogenes* growth inhibition produced as a result of an antagonistic interaction with *Bacillus* spp.

Table 6.1 *Bacillus* isolates which exhibited an inhibitory activity (mm) against different colony forms of *L. monocytogenes* via the direct stab antagonistic method on TSYEA plates.

Identification	Source	Stock No.	BCET-RPLA Reaction‡	Zone of inhibition (mm)		
				NCTC 11994[a]	NCTC 9863[b]	NRB2[c]
B. mycoides	A16 31/03/93	1		1.1	1.0	2.1
B. cereus II	No.1 29/09/93	3	+	1.3	1.2	1.5
B. cereus I	NCTC 11143	7		3.8	3.5	2.8
B. cereus II	No.2 29/09/93	8	-	2.8	3.0	2.1
B. mycoides	No.1 Ruchill Hosp.	15		1.1	1.2	1.8
B. mycoides	*2 Apt 31/03/93	17		1.5	0.8	1.0
B. cereus I	B1. 17/04/93	21		0.5	NZD	1.1
B. subtilis	C12. 2/04/93	23†		2.9	3.1	4.2
B. cereus I	B16. 31/03/93	24		3.7	4.2	3.7
B. cereus II	No.1 15/05/93	27	-	4.1	3.5	3.6
B. cereus II	A1. FM2 15/7/93	28	-	3.4	3.6	3.1
B. cereus II	B18. 31/03/93	30	+	3.0	2.8	1.4
B. cereus II	B11 31/03/93	31	+	0.5	0.5	NZD
B. megaterium	B4. 31/03/93	35		3.3	3.3	3.5
B. cereus II	No.14 29/10/93	38	-	4.8	4.1	4.5
B. cereus I	No. 21 12/10/93	44		NZD	NZD	1.2
B. cereus I	No.5 12/10/93	45		NZD	NZD	1.0
B. cereus II	No.6 20/10/93	48	-	1.5	1.5	1.5
B. mycoides	No.7.08/09/93	58		4.2	3.9	3.5
B. cereus II	No.7a 14/09/93	59	-	4.3	3.8	3.1
B. cereus II	No.4 29/10/93	62	+	NZD	NZD	0.5
B. cereus I	No.8 20/10/93	63		1.8	2.0	2.1
B. cereus I	No.8 14/12/93	65		3.2	2.2	5.0
B. cereus II	No.6 14/12/93	70	-	1.8	1.6	1.8
B. cereus II	A1 17/02/94	92	-	3.0	3.0	3.4
B. cereus II	No.2. Yorkhill Hosp	93	-	2.5	2.6	3.0
B. cereus II	No.7 Yorkhill Hosp	99	-	2.3	2.0	2.1
B. subtilis	No.9 Yorkhill Hosp	104†		1.1	NZD	1.4
B. cereus I	No.3 15/07/94	107		1.3	1.5	2.0
B. cereus I	No.4 15/07/94	108		1.0	1.0	1.5
B. cereus II	A2. 15/07/94	113	-	3.0	3.1	3.3
B. amyloliquefaciens	NCIMB 12077	118†		2.0	0.5	2.0
B. subtilis	A13. 17/05/94	132†		3.1	3.3	3.0
B. subtilis	A14. 17/05/94	133†		3.7	3.5	4.3
B. mycoides	A29. 17/05/94	148		2.5	1.9	0.5
B. licheniformis	A5 03/07/94	152†		2.0	0.5	NZD
B. licheniformis	A9 03/07/94	167†		2.3	2.4	2.6
B. cereus II	A15 03/07/94	168	-	1.6	1.6	1.5
B.amyloliquefaciens	A16 03/07/94	170†		4.0	3.5	4.1
B. subtilis	A7. 15/07/94	173†		3.2	3.5	3.1
L. monocytogenes	NCTC 11994	Control		NZD	NZD	NZD
L. monocytogenes	NCTC 9863	Control		NZD	NZD	NZD
L. monocytogenes	NRB2	Control		NZD	NZD	NZD
B. cereus II	NCTC 11145	Control	+	NZD	NZD	NZD
B. mycoides	NCTC 926	Control		NZD	NZD	NZD
B. subtilis	NCTC 3610	Control		NZD	NZD	NZD
B. pumilus	NCTC 10337	Control		NZD	NZD	NZD
B. brevis	NCTC 2611	Control		NZD	NZD	NZD
B. licheniformis	NCTC 10341	Control		NZD	NZD	NZD
B. laterosporus	NCTC 7579	Control		NZD	NZD	NZD
B. lentus	NCTC 4824	Control		NZD	NZD	NZD
B. sphaericus	NCTC 7582	Control		NZD	NZD	NZD
B. amyloliquefaciens	NCIMB 10785	Control		NZD	NZD	NZD
B. circulans	NCTC 9432	Control		NZD	NZD	NZD
B. megaterium	NCTC 6005	Control		NZD	NZD	NZD

† Illustrates the Bacillus spp. which had to be stored at an assay temperature of 25°C in order to detect the zone of inhibition.
‡ Represents the strains of B. cereus II which had demonstrated a positive Reverse Phase Latex Agglutination (RPLA) test reaction for diarrhoeal enterotoxin production.
[a,b,c] Are *L. monocytogenes* NCTC 11994, NCTC 9863 and NRB2 respectively.

In order to establish whether these *Bacillus* spp. (namely 9 members comprising of *B. licheniformis, B. subtilis* and/or *B. amyloliquefaciens*) exhibited an antagonistic activity, the direct stab inoculation technique was repeated at the lower assay temperature of 30°C. This study revealed that all 9 members of this *Bacillus subtilis* subgroup were capable of inhibiting the growth of both colony forms of *L. monocytogenes*, as distinct zones of inhibition were identified at the assay temperature of 30°C (these *Bacillus spp.* can be identified by the symbol † in Table 6.1).

The results of this preliminary screen also revealed that, while 22.5% of the *Bacillus* species isolated from infant milk formula demonstrated varying degrees of antagonism towards both rough and smooth forms of *L. monocytogenes*, the control *Bacillus* test strains (supplied by the National Collection of Typed Cultures) did not inhibit their development (i.e. no zone of inhibition detected- NZD). Indeed, while certain test organisms showed a varied antagonistic response to the different surface culture forms of *L. monocytogenes*, there was no overall significant difference (Wilcoxon Ranked Sign Test-P0.05 level) between the 3 indicator organisms in relation to the level of antagonism experienced.

6.1.1.2 Confirmation that the previously employed conditions were optimal for the subsequent identification of all antagonistic *Bacillus spp.*

In order to confirm that the conditions employed in the above simultaneous screening study were satisfactory for the subsequent identification of presumptive antagonistic *Bacillus* (i.e. a *Listeria* inoculum level of 10^5 cells ml^{-1} on TSYEA medium), the direct stab inoculation technique was repeated using a wider dilution range of smooth and rough form *L. monocytogenes* (i.e. 10^{-1}, 10^{-3}, 10^{-5} dilutions) in addition to using a greater variety of assay media (TSYEA, BHIA and BA).

The 3 indicator organisms (*L. monocytogenes* NCTC 11994, NCTC 9863, NRB2) were cultured to a homogeneous cell population level (approximately 10^9 CFU ml^{-1}) as mentioned above. A tenfold dilution of these indicator cultures was made and 0.1 ml aliqouts of the 10^{-1}, 10^{-3}, and 10^{-5} dilutions were spread over the surface of a TSYEA, BHIA and BA plate to give a total aerobic mesophilic count of

approximately 10^7, 10^5 and 10^3 CFU ml^{-1} respectively. A total aerobic mesophilic count was carried out to confirm the *Listeria* cell concentration on each of these seeded plating media.

Immediately after, the 40 short listed *Bacillus* spp. (previously shown to exhibit a strong antagonistic activity against *L. monocytogenes*) were separately seeded onto the above mentioned plating media via the conventional stab inoculation technique (section 3.14.2) and a maximum of five inoculations of the test organism *(Bacillus spp.)*, spaced 2 cm apart on duplicate plates were carried out (Table 6.2). This direct stab antagonistic method was repeated for both the smooth (NCTC 11994, NCTC 9863) and rough (NRB2) colony forms of *L. monocytogenes*. The plates were then incubated at 30°C for 24 hours. The result of the antagonistic interaction between *Bacillus* spp. and the *Listeria* colony forms is illustrated in Table 6.2, where the inhibition zone (mm) produced by *Bacillus* against *L. monocytogenes* is presented as an average measurement made of the 5 stab inoculations on the duplicate plates (*i.e.* the mean of 10 inhibition zone measurements).

Indeed, the results of this direct antagonistic interaction between the test organisms (*Bacillus spp.*) and the seeded indicator organism *(L. monocytogenes)* revealed that the inhibitory activity of *Bacillus* appears to be proportional to the population of *Listeria* seeded on the plate. Where higher numbers of *Listeria* (10^7 cells ml^{-1}) were distributed evenly over the assay plate, a narrow inhibition zone around the test organism was observed (i.e. zone of clearing < 2.0 mm). In some instances a zone of inhibition was not observed at this upper *Listeria* dilution level, while an observation of the plates seeded with lower dilutions of *Listeria* demonstrated moderate antagonistic interactions. Conversely, a strong inhibition (zone of clearing ~6 mm) was observed when low numbers of *Listeria* (e.g. 10^3 cells ml^{-1}) were tested. At the lower *Listeria* dilutions certain areas of the seeded agar plate surface were only sparsely populated with *Listeria* colonies and it was not possible to confidently establish whether or not the area of clearing was due to an antagonistic interaction or due to an uneven distribution of the indicator organism across the agar surface.

Particularly well defined diffuse zones were observed when 10^5 *L. monocytogenes* cells were spread evenly across the surface of the TSYEA plates prior to the stab

inoculation of *Bacillus* spp. This resulted in a zone of *Listeria* clearing between 2 to 5 mm. Additionally, as the test organisms generally exhibited a smaller colony size when they were cultivated at 30°C, this facilitated the appearance of distinct measurable zones of inhibition. The level of antagonism (represented by the size of the inhibition zone) between the separate *Bacillus spp.* and the 3 different dilution levels of both *L. monocytogenes* colony forms is illustrated in Table 6.2.

Table 6.2 Illustrates the direct antagonistic interaction between the *Bacillus* spp and different dilution levels of rough and smooth form *L. monocytogenes* which had been seeded onto the surface of TSYEA plates.

Identification	Stock No.	Zone of inhibition (mm)								
		Smooth form (NCTC 11994)			Smooth form (NCTC 9863)			Rough form (NCTC NRB2)		
		$10^{7\dagger}$	10^{5}	10^{3}	10^{7}	10^{5}	10^{3}	10^{7}	10^{5}	10^{3}
B. mycoides	1	NZD	NZD	NZD	NZD	1.0	3.3	0.4	2.0	3.3
B. cereus II	3	0.3	1.1	3.0	0.4	1.3	3.5	0.6	1.5	3.6
B. cereus I	7	1.1	3.9	4.35	0.6	3.4	4.4	1.1	2.8	4.25
B. cereus II	8	1.4	3.0	4.6	1.4	3.0	4.5	0.9	2.1	4.1
B. mycoides	15	0.3	1.2	3.5	0.3	1.2	3.1	0.5	1.9	3.5
B. mycoides	17	0.2	1.5	3.5	NZD	0.6	3.0	0.3	1.0	3.0
B. cereus I	21	NZD	0.5	3.1	NZD	NZD	3.0	NZD	1.0	3.5
B. subtilis	23	1.7	3.0	4.7	1.5	3.1	4.2	2.2	4.3	5.9
B. cereus I	24	1.8	3.8	5.5	2.1	4.0	5.4	1.6	3.6	5.5
B. cereus II	27	1.9	4.0	4.6	1.7	3.7	4.4	2.1	3.7	5.2
B. cereus II	28	1.7	3.6	5.1	1.7	3.6	5.0	1.7	3.2	4.6
B. cereus II	30	1.5	3.1	4.4	1.4	2.6	4.1	NZD	1.0	3.3
B. cereus II	31	NZD	0.5	2.9	NZD	0.6	3.0	NZD	0.4	2.9
B. megaterium	35	1.8	3.5	5.0	1.1	3.3	4.6	1.8	3.6	5.1
B. cereus I	38	2.3	5.0	6.1	2.0	3.9	5.1	1.8	4.5	5.2
B. cereus I	44	NZD	NZD	NZD	NZD	NZD	NZD	NZD	1.0	2.9
B. cereus I	45	NZD	NZD	NZD	NZD	NZD	NZD	NZD	1.0	3.0
B. cereus II	48	0.5	1.5	3.5	0.6	1.7	3.9	0.6	1.5	4.0
B. mycoides	58	1.8	4.1	5.4	1.7	3.9	5.1	1.5	3.5	4.6
B. cereus II	59	1.9	4.6	5.3	1.7	4.0	5.2	2.0	3.3	5.1
B. cereus II	62	NZD	0.3	3.0	NZD	0.3	2.9	NZD	0.5	3.0
B. cereus I	63	0.3	1.8	3.6	0.3	1.8	4.0	0.5	2.1	4.2
B. cereus I	65	1.6	3.1	5.2	0.6	1.9	3.3	2.3	5.6	6.0
B. cereus II	70	1.1	2.0	3.6	1.0	1.6	3.1	1.3	2.2	3.5
B. cereus II	92	1.5	3.1	4.4	1.6	3.0	4.5	2.0	3.8	5.0
B. cereus II	93	2.0	3.5	4.8	1.7	3.1	4.2	1.7	3.5	4.6
B. cereus II	99	1.6	2.3	4.1	1.2	1.8	3.8	1.5	2.5	3.9
B. subtilis	104	NZD	1.0	3.1	NZD	NZD	1.5	NZD	0.9	2.7
B. cereus I	107	NZD	1.3	2.4	0.5	1.6	3.2	1.0	2.0	3.5
B. cereus I	108	NZD	1.0	2.1	NZD	0.5	2.1	NZD	1.6	2.9
B. cereus II	113	2.1	3.5	5.1	1.9	3.3	4.8	2.0	3.5	5.2
B.amyloliquefaciens	118	1.4	2.0	3.5	NZD	0.3	1.9	1.1	1.5	2.5
B. subtilis	132	2.0	3.2	4.4	2.1	3.6	5.3	2.2	3.5	5.8
B. subtilis	133	1.6	3.8	4.8	1.5	3.5	4.6	2.3	4.5	6.1
B. mycoides	148	1.1	2.5	3.5	0.7	1.9	3.3	NZD	0.5	2.8
B. licheniformis	152	1.5	2.7	4.2	NZD	0.3	2.1	NZD	1.1	2.5
B. licheniformis	167	1.6	2.4	3.7	1.5	2.4	3.5	1.8	2.6	3.4
B. cereus II	168	0.5	1.6	2.8	NZD	1.1	2.5	NZD	1.2	2.8
B. amyl oliquefaciens	170	2.2	4.2	4.7	1.9	3.3	4.4	2.0	4.0	4.2
B.subtilis	173	1.6	3.1	4.1	1.8	3.5	4.3	1.6	3.0	4.0
MEAN		1.125	2.40	4.43	1.05	2.09	4.52	1.54	2.88	4.05
S.D		0.79	1.40	4.03	1.20	1.37	5.91	2.57	2.72	1.10

† Refers to the actual Listeria cell concenration (CFU ml^{-1}) spread over the surface of TSYEA plates

In order to establish whether or not the composition of the culture medium influenced the level of antagonism experienced by *Listeria* when grown simultaneously with *Bacillus*, the direct stab antagonism method was repeated using 6 randomly selected *Bacillus* spp which had previously exhibited a strong inhibitory activity against *L. monocytogenes*.

The same indicator organisms were cultivated under similar conditions as mentioned above and tenfold dilutions were subsequently made after the 20 hour incubation period. A 0.1 ml aliquot of the 10^{-1}, 10^{-3} and 10^{-5} dilutions were spread over the surface of BHIA, TSYEA and BA plates. A total aerobic mesophilic count (CFU ml^{-1}) was determined for each dilution plate in order to confirm the level of indicator organism (*Listeria*) present at the point of test culture (*Bacillus*) inoculation. The seeded plates were allowed to dry for 30 minutes prior to the stab inoculation of the test organism. The test organisms consisted of *Bacillus* spp. test culture number's 7, 28, 58, 59, 133, and number 170 (all taken from the infant milk formulae culture collection). Each duplicate assay plate was stab inoculated in 5 positions at a distance of 2 cm apart and the plates were incubated at 30°C for 24 hours.

After this incubation period, the zone of inhibition was measured (mm) and averaged (regardless of whether they came from one or more assay plates) for each combination of test organism, concentration of indicator organism, and the type of culture media employed. The results of this antagonistic interaction are illustrated in Table 6.3.

The information presented in Table 6.3 reveals that TSYEA appears to be the most suitable assay medium (P0.05) for the purpose of demonstrating antagonistic interactions between the test organisms (*Bacillus* spp.) and *L. monocytogenes* (both colony forms), as greater zones of *Listeria* clearing were observed on this assay medium compared to the other two plating media.

Attempts to measure the zones of inhibition on BA, produced as a result of antagonistic interactions between certain *Bacillus* spp. (e.g. *B. cereus* and *B. amyloliquefaciens*) and *Listeria*, was obscured or hampered due to the strong β-haemolysis (i.e. destruction of red blood cells) produced by these test organism in Blood Agar. While a greater level of antagonism was observed for test and indicator

organisms seeded on TSYEA plates compared to either BA and/or BHIA, there appearred to be no significant difference between the antagonistic interactions carried out on the latter two assay media (P0.05).

Table 6.3 Illustrates the zones of inhibition (mm) produced as a result of a simultaneous antagonistic interaction between *Bacillus* spp. and *L. monocytogenes* on three separate plating media.

Stock	Culture	Smooth form (NCTC 11994)			Smooth form (NCTC 9863)			Rough form (NRB2)		
NO.	Medium	$10^{7\dagger}$	10^{5}	10^{3}	10^{7}	10^{5}	10^{3}	10^{7}	10^{5}	10^{3}
7	TSYEA	1.1	3.9	4.3	0.6	3.4	4.4	1.2	2.8	4.1
28	TSYEA	1.7	3.6	5.1	1.5	3.5	4.8	1.7	3.2	4.9
58	TSYEA	1.8	4.1	5.3	2.0	3.9	5.5	1.5	3.5	4.6
59	TSYEA	1.9	4.5	5.1	1.6	4.0	5.4	2.0	3.3	5.2
133	TSYEA	1.5	3.8	4.7	1.1	3.5	4.6	2.3	4.3	5.8
170	TSYEA	2.1	4.1	4.8	1.5	3.4	4.3	2.3	4.2	4.3
	Mean	*1.68*	*4.0*	*4.83*	*1.38*	*3.61*	*4.83*	*1.83*	*3.55*	*4.81*
	S.D	*0.34*	*0.30*	*0.34*	*0.48*	*0.26*	*0.51*	*0.45*	*0.59*	*0.62*
7	BHIA	0.5	3.0	3.7	0.3	3.3	3.8	0.6	2.6	3.6
28	BHIA	0.5	2.9	4.5	1.1	2.7	4.3	0.5	2.6	4.2
58	BHIA	1.0	3.4	4.7	1.5	3.3	4.5	1.1	2.9	4.2
59	BHIA	1.2	4.4	5.0	1.2	3.8	5.1	1.3	2.9	4.9
133	BHIA	1.1	4.0	4.2	1.1	3.6	4.0	1.0	3.8	4.5
170	BHIA	1.5	4.1	4.1	1.4	3.3	3.9	1.3	3.8	4.2
	Mean	*0.96*	*3.63*	*4.36*	*1.1*	*3.33*	*4.26*	*0.96*	*3.1*	*4.26*
	S.D	*0.40*	*0.62*	*0.46*	*0.42*	*0.37*	*0.48*	*0.34*	*0.56*	*0.43*
7	BA	0.5	2.0	3.3	NZD	2.1	3.1	NZD	1.5	3.2
28	BA	0.4	2.2	3.8	NZD	2.4	3.2	NZD	2.2	3.7
58	BA	1.1	3.3	4.8	1.6	3.0	4.5	1.5	3.2	4.3
59	BA	NZD	2.4	4.2	0.4	1.9	4.3	0.6	1.8	4.3
133	BA	1.0	3.1	3.6	1.0	2.9	4.1	1.2	2.5	3.9
170	BA	1.3	2.6	3.5	1.1	2.8	3.5	1.0	2.3	3.8
	Mean	*0.72*	*2.6*	*3.87*	*0.68*	*2.53*	*3.78*	*0.71*	*2.25*	*3.87*
	S.D	*0.50*	*0.51*	*0.55*	*0.65*	*0.46*	*0.59*	*0.62*	*0.58*	*0.41*

† Represents the number of *Listeria* cells seeded on each plating medium.

The results from this supplementary antagonistic study confirmed that the initial screening (using the stab inoculation technique of TSYEA plates) technique was satisfactory for the purpose of identifying *Bacillus* spp. (from the infant milk formulae culture collection) demonstrating antagonism against different colony forms of *L. monocytogenes*. In addition, this preliminary screen also illustrated that of the 178 test organisms challenged against the two colony forms of *Listeria monocytogenes*, only 3 out of the resulting 40 antagonistic *Bacillus spp.* (i.e. 7.5%) did not inhibit all 3 strains of *L. monocytogenes*. It was shown that there was no significant difference

(P0.05) between the various morphological forms of *L. monocytogenes* when it came to the overall interaction with the 178 test organisms.

6.1.1.3 Deferred antagonism

Confirmation of the antagonistic activity demonstrated by *Bacillus* spp. against different morphological colony forms of *L. monocytogenes* on solid media was attempted via the deferred method (see section 3.14.3). Using the conventional technique, the test organisms were stab inoculated onto the surface of TSYEA and BHIA plates. A maximum of 5 inoculations of the test organism (i.e. each of the 40 *Bacillus* spp. previously shown to be strongly antagonistic), spaced approximately 2 cm apart, were aseptically performed per plate. These assay plates were then incubated for 18-24 hours at 30°C.

After this incubation period, a 0.1 ml aliquot of a 24 hour *L. monocytogenes* culture (using a 10^{-3} dilution) grown in TSYEB as described above, was aseptically inoculated into separate sterile disposable universal bottles containing 5 ml of TSYEA or BHIA soft agar (0.7%). The soft agar containing the indicator organism was carefully overlaid on the surface of the agar which had been previously seeded with *Bacillus spp.*

The inhibitory process was detected in the 2 assay media, where the bottom TSYEA layer (which had been seeded with the test organism) was overlaid with either TSYEA or BHIA containing approximately 10^5 *Listeria* cells ml^{-1}. The combination of stab inoculation and assay temperature (30°C) was sufficient to observe a zone of distinct *Listeria* inhibition produced by the test organism. However, the conventional method did not always show reproducible results. Often, it was noted that when soft agar (containing *L. monocytogenes*), was overlaid on the agar surface in which the test organism (*Bacillus*) had grown, certain *Bacillus* spp. tended to spread and as a result a clear zone of inhibition could not be easily identified (Table 6.4). This deferred antagonistic techniques did confirm that the initial direct or simultaneous antagonist screening method (viz. stab inoculation) was sufficient to identify potential antagonistic *Bacillus* spp.

The inhibitory activity produced as a result of an antagonistic interaction between *Bacillus spp* (i.e. for the short listed 40 antagonistic *Bacillus* spp.) and *L. monocytogenes* was also investigated in the absence of living *Bacillus* cells. This was carried out in order to confirm whether the inhibitory activity was caused by the presence of substances which had possibly diffused into the media.

For this purpose, the test organism (*Bacillus spp.*) was aseptically removed using a membrane filter (0.2µm) on which the test organism had grown, leaving a cell free area on the agar surface where the membrane had been initially placed (section 3.14.4).

After eliminating the live *Bacillus* cells, the two pleomorphic surface culture forms of *L. monocytogenes* (NCTC 11994 and NRB2) were carefully inoculated by the overlay technique. All procedures were carried out as described in the section 3.14.3.1). After seeding the *Listeria* cells (either the smooth or the rough colony form), the assay plates were then incubated for 20 hours at 30°C. Following this incubation period, inhibition was observed by the presence of a clear zone in the area which had been previously occupied by the *Bacillus* colony or the membrane filter. Quantification of the level of antagonism was not determined, as the purpose of the study was to simply reveal whether or not the *Listeria* cells were inhibited by the test organisms (i.e. absence or presence).

Results from this deferred procedure revealed an inhibition of *Listeria* (as detected by a zone of clearing) via the membrane filter method. This indicated that the antagonistic interaction exhibited by *Bacillus spp.* against *L. monocytogenes* was not due to the mere presence of the *Bacillus* cells, but rather it was possibly due to the liberation of some metabolic product from the cell into and/or alteration of the surrounding culture medium).

6.1.2 Antagonistic assay in semisolid culture media (i.e. soft agar).

The antagonistic interaction between *Bacillus spp.* and morphological colony variants of *L. monocytogenes* was also examined using a direct method in semisolid media. The semi-solid TSYEA and BHIA culture media were prepared by the addition of

0.7% of bacteriological agar. The level of antagonism exhibited by *Bacillus* against different inoculum levels of *L. monocytogenes* was evaluated in the semi-solid culture media.

Table 6.4 Deferred antagonistic technique- where the zones of inhibition (mm) produced as a result of a *Bacillus spp.* antagonistic interaction against 3 strains of *L. monocytogenes* which were overlaid in either TSYEA or BHIA (approximately 10^5 cells ml^{-1}) onto a bottom TSYEA layer.

Stock No. (Bacillus spp.)	TSYEA Overlay			BHIA Overlay		
	NCTC 11994	NCTC 9863	NRB2	NCTC 11994	NCTC 9863	NRB2
1	NZD*	1.6*	2.5*	NZD	1.1*	2.1*
3	1.9*	2.6	2.5*	1.3	2.5	1.4*
7	4.2	3.6*	3.0*	3.7	3.5*	2.4
8	3.0*	2.8	2.3*	2.6*	2.7	2.0*
15	1.1*	1.0	1.3*	1.3	1.3*	1.6*
17	1.4*	*	0.8*	*	1.0*	*
21	0.8	NZD*	1.4*	0.5*	-	1.2
23	2.6*	3.3*	*	2.2*	2.6*	2.5*
24	3.6	3.6	3.5*	3.0	2.8*	3.1*
27	4.4*	3.9*	3.4*	3.1*	3.0*	2.6*
28	3.6*	3.8*	3.5*	3.2	2.8	2.7
30	3.6*	3.1*	1.1	2.8*	2.6*	0.8*
31	0.5*	1.0*	0.5	0.5	NZD*	NZD
35	4.1*	3.6*	3.5	3.6	3.7*	4.0
38	4.8*	4.2	4.6	3.6*	3.6	3.45
44	NZD	NZD*	0.5	NZD*	NZD	NZD
45	NZD	NZD	1.6	NZD	NZD	0.5
48	2.1	1.8	1.5	2.0	2.0*	1.3
58	4.1*	3.5*	2.9*	3.6*	3.2*	2.2*
59	4.0*	4.1*	2.9	3.0	2.9*	2.0*
62	0.5	0.5	NZD	NZD	0.5*	0.5
63	1.8*	2.0	2.4	1.8*	1.8	2.0*
65	3.5*	2.5*	5.0	3.0*	2.4	4.1*
70	2.5*	1.8*	2.2*	2.0	1.7*	2.1
92	3.4*	3.0*	3.6	3.1	3.0*	3.3
93	4.0*	4.0	4.0	3.3	3.4	3.4
99	2.4	*	2.3	2.1*	1.8	2.4
104	1.5*	NZD	1.0	1.5	NZD	1.0
107	1.5	1.5	2.0*	1.0*	1.0	1.0
108	0.5*	0.5*	1.0*	NZD	0.5*	1.0
113	3.7*	3.5	3.5*	3.2	3.1	2.8
118	*	*	*	1.5*	3.5*	*
132	3.5*	3.5*	3.0*	2.8*	*	3.0*
133	3.5*	*	4.0*	3.1*	3.0*	*
148	3.0*	2.0*	0.5*	2.0*	1.5*	NZD
152	2.0*	NZD*	NZD*	1.5*	NZD*	NZD
167	0.5*	0.5*	0.5*	0.5*	0.5*	NZD*
168	1.5	1.5*	1.0*	1.0	1.0*	NZD
170	4.0*	3.0*	3.0*	3.0*	2.5*	2.0*
173	1.0*	1.5*	2.5*	1.0*	1.0*	2.0*

* Illustrates spreading of test organism, making the zone of inhibition (mm) difficult to measure.

Owing to the large number of *Bacillus spp.* exhibiting various degrees of antagonism against both colony forms of *Listeria* (Tables 6.1 and 6.2), it was not feasible to

challenge every antagonistic *Bacillus spp.* against the different inoculum levels of *L. monocytogenes* (smooth and rough colony form).

Therefore, a short list of 8 antagonistic *Bacillus* spp. was made. These test organisms shared two common characteristics: being able to grow at refrigeration temperatures (≤8°C, as determined in section 6) and/or producing an antagonistic response when cultivated with either colony form of *L. monocytogenes*. The following *Bacillus* isolates satisfied these criteria; *B. cereus II* (No. 3), *B. mycoides* (No.15), *B. cereus I* (No. 29), *B. megaterium* (No. 35), *B. cereus* 1 (No. 44), *B. mycoides* (No.58), *B. subtilis* (No.132), *B. subtilis* (No. 133) and *B. amyloliquefaciens* (No. 170) and were employed as test organisms. The test control organisms used throughout this study were *B. cereus II* (NCTC 11145) and *L. monocytogenes* (NCTC 11994).

The assay was carried out by initially cultivating the test and indicator organisms in TSYEB at 35°C under gyrotary conditions (110 rpm). After a 20 hour cultivation period , a 0.1 ml aliquot of the neat and tenfold dilutions thereof (10^{-1} to 10^{-5}) were aseptically inoculated into sterile transparent universal bottles containing 10 ml of either soft TSYEA or BHIA agar (0.7%). The same procedure was repeated for the rough colony form of *L. monocytogenes* (NRB2). Upon solidification of these agars, a 0.1ml aliquot of a 20 hour test culture of *Bacillus* (cultivated in TSYEB) was inoculated onto the agar surfaces.

The bottles (or tubes) were incubated at 30°C and 8°C for 24/48 hours and 6 days respectively. Growth inhibition of *L. monocytogenes* was confirmed by the appearance of a clear volume or a reduction in turbidity below the surface of the media (Figure 6.2).

Therefore, the extent of *Listeria* inhibition was defined in terms of the reduction in turbidity (expressed in terms of volume- mm^3) below the agar surface, the greater the inhibition, the larger the reduction in turbidity (i.e. volume of cells cleared). This volume of *Listeria* inhibition (clearance) can be calculated from the formulae $\pi r^2 h$, where h is the distance (mm) from the surface of the agar to the end of the clear zone and r is the radius (mm) across the surface of the agar (Figure 6.2).

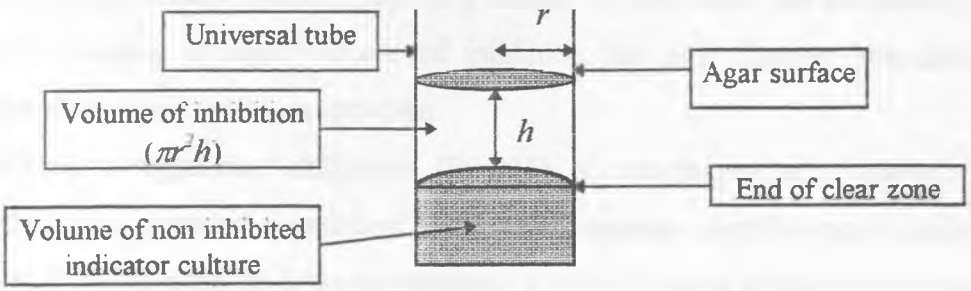


Figure 6.2 A diagrammatic representation of the inhibition of *L. monocytogenes* (expressed as a volume of clearance below the surface of the agar which had been seeded with the indicator organism) in soft agar.

In this experiment, volumes of *Listeria* inhibition were observed in both assay media. Indeed, similar to the inhibition process in solid media, the antagonistic effect of the various test *Bacillus* spp. was *inversely* proportional to the population of the *Listeria* cells present in the semi-solid medium. Indeed, this phenomenon is clearly illustrated in Figure 6.3, where the level of antagonism produced by *Bacillus* is influenced by the concentration of *Listeria* cells present and the type of assay medium employed.

As illustrated in Figure 6.3, the greater the number of *L. monocytogenes* cells present, the smaller the volume of inhibition experienced. Moreover, it does appear that a suspended *Listeria* cell population of 10^5 CFU ml^{-1} is optimum for the identification and subsequent quantification of antagonistic interactions between *Bacillus* and *Listeria* in semi-soft agar media. The deferred inhibition process is shown in Figure 6.4, where *B. mycoides* (No.58) had been seeded onto TSYEA which had supported the growth of a 24 hour culture of *L. monocytogenes* (No.77) at 30°C.

The inhibition process exhibited by the 8 *Bacillus* spp. against different inoculum sizes of S and R-colony forms of *L. monocytogenes* (24 hours at 30°C) is illustrated in Table 6.5. It is quite apparent that the volume of *Listeria* growth inhibition is related to the actual concentration of *Listeria* cells present in the soft agar medium.

Furthermore, while the composition of the culture media employed did not appear to significantly influence (P0.05) the volume of growth inhibition experienced by the *Listeria* cells, certain *Bacillus* spp. (e.g. No. 170) did affect the suspended *Listeria* cells by causing a larger volume of inhibition (at each *Listeria* inoculum level), irrespective of the media composition.

Therefore, a significant difference (P0.005) in the degree of *L. monocytogenes* inhibition was revealed, depending on the test organism (*Bacillus* type) employed. In addition, the variation in *L. monocytogenes's* morphological appearance did not make this indicator organism more susceptible to antagonism or result in a greater level of inhibition on interaction with antagonistic *Bacillus* spp.

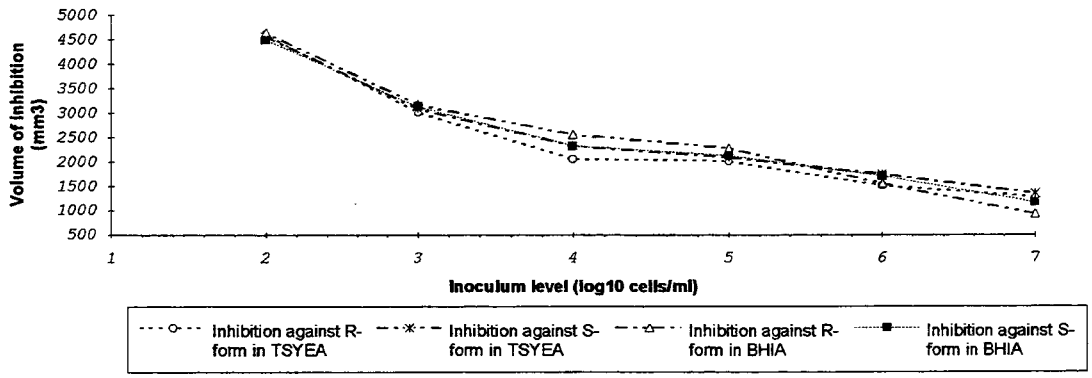


Figure 6.3 Direct Antagonism Method: Effect of *Listeria* cell inoculum level and different culture media on the growth inhibition (mm^3) of rough and smooth colony forms of *L. monocytogenes* by *B. subtilis* (No.133).

The volume of *Listeria* inhibition (i.e. both colony forms) exhibited in BHI soft agar is not illustrated in this study, as application of statistical analysis (Wilcoxon Ranked Sign Test) showed that there was no significant difference (P0.05) between culture media in relation to the level of inhibition experienced at each *Listeria* inoculum level. However, the antagonistic activity produced by *Bacillus amyloliquefaciens* (No. 170) did result in a significantly greater amount of growth inhibition (P0.05) at each *Listeria* inoculum level in BHIA, compared to the same interaction in TSYEA at 30°C

(Figure 6.5). As there was no significant difference (P0.05) in the degree of growth inhibition experienced by the 2 colony forms of *Listeria*, only the antagonistic interaction exhibited between *B. amyloliquefaciens* and the smooth form of *L. monocytogenes* was selected for illustration (Figure 6.5).

Table 6.5 Direct antagonistic technique- volume of inhibition (mm^3) experienced by S and R form *Listeria* cells at different inoculum levels (cells ml^{-1}) on interaction with different *Bacillus spp.* in soft TSYEA agar at 30°C.

Stock No.	Volume (mm^3) of inhibition at different *Listeria* inoculum levels (cells ml^{-1} TSYEA soft agar)											
	10^7		10^6		10^5		10^4		10^3		10^2	
	Smooth*	Rough+	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough
3	NZD¶	NZD	NZD	NZD	NZD	NZD	193	387	774	774	1548	1856
15	NZD	NZD	NZD	NZD	193	387	541	619	1161	1277	2012	2090
29	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	387	426	774	890
35	348	387	1161	987	1509	1746	1896	1780	2709	2787	3444	3406
44	426	387	1354	1085	1548	1896	1974	2128	2709	2903	3096	3367
58	1045	774	1625	1374	1896	1856	2322	2128	2846	3019	3290	3212
132	1083	987	1548	1470	1856	1741	1354	2051	2980	2941	4451	4451
133	1277	1354	1509	1741	2012	2090	2051	2322	3019	3096	4606	4567
170	387	387	1161	1199	1277	1432	1935	1856	2825	2709	3329	3290
Control	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	387	387	696	774

* Smooth colony form of *L. monocytogenes* (NCTC 11994)

+ Rough colony form of *L. monocytogenes* (NRB2)

¶ No inhibition zone detected

The effect of *Listeria* inoculum level, assay temperature and type of antagonistic *Bacillus* spp. employed on the subsequent inhibition (mm^3) of both colony forms of *L. monocytogenes* in TSYEA and BHIA is illustrated in Table 6.6. Examination of the findings in this table revealed that certain test organisms (i.e. No.'s 35, 58, 132, 133, and 170) exhibited superior levels of antagonism against the S and R form *Listeria* and at 8°C and 30°C.

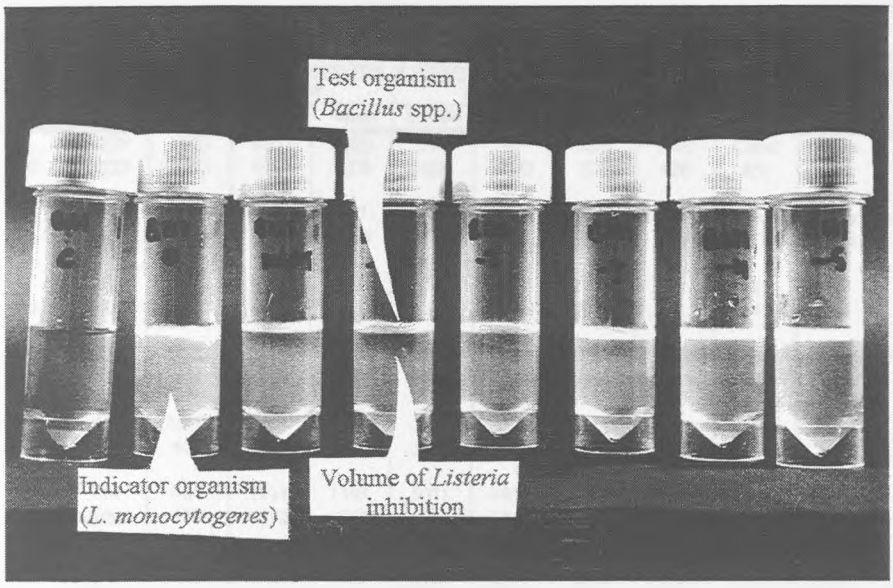


Figure 6.4 Deferred antagonistic method- inhibition of *L. monocytogenes* (NCTC 11994) by *B. mycoides* (No. 58) at 30°C, where *Listeria* was suspended in BHI soft agar at different inoculum levels.

Therefore, under the present set of conditions there appears to be a variation in the ability of the individual *Bacillus* spp. to obtain the same level of growth inhibition at each indicator inoculum level.

A greater level of *Listeria* growth inhibition was achieved when the antagonistic assay was performed at the reduced storage temperature of 8°C compared to that of 30°C described above. Indeed, when both sets of assay plates were stored for 6 days at their respective temperatures, a greater volumetric reduction (mm^3) in *Listeria* cells occurred when the antagonistic interaction was performed at the near refrigeration temperature of 8°C compared to that of 30°C. To illustrate the effect of storage temperature on the subsequent inhibition of *Listeria*, the results of the 6 day inhibition process at both assay temperatures are shown in Table 6.6.

Table 6.6 Direct Antagonism Technique (Semisolid medium): effect of inoculum level (*Listeria* cells ml^{-1}) and assay temperature (°C) on the inhibition (mm^3) of different colony forms of *L. monocytogenes* (where *Bacillus spp.* were the test organisms).

Stock No.	Assay Temp. (°C)	Volume (mm^3) of inhibition at different inoculum levels (Listeria cell ml^{-1} on TSYEA soft agar)											
		10^7		10^6		10^5		10^4		10^3		10^2	
		Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough
3	8	NZD‡	NZD	NZD	NZD	774	987	3667	3367	Clear†	Clear	Clear	Clear
	30	NZD	NZD	NZD	NZD	NZD	NZD	270	426	851	774	11625	1856
15	8	NZD	NZD	541	387	2322	1741	4264	4064	Clear	Clear	Clear	Clear
	30	NZD	NZD	NZD	NZD	270	426	580	619	1270	1354	2012	2090
29	8	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	425	580	967	774
	30	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	445	503	851	948
35	8	NZD	NZD	580	387	3096	2903	Clear	Clear	Clear	Clear	Clear	Clear
	30	425	464	1227	1180	1651	1851	1951	2010	3450	3226	3886	3665
58	8	812	NZD	1161	580	3677	3328	Clear	Clear	Clear	Clear	Clear	Clear
	30	1086	822	1654	1540	2080	1956	2865	2678	3674	3657	3854	3954
132	8	774	387	1741	1393	5031	4837	Clear	Clear	Clear	Clear	Clear	Clear
	30	1083	987	1592	1560	1980	1944	1736	2240	3210	3350	4480	4582
133	8	580	193	1548	1625	4837	5224	Clear	Clear	Clear	Clear	Clear	Clear
	30	1345	1356	1574	1786	2402	2650	2874	2952	3556	3493	4704	4665
170	8	967	1201	2515	2747	3637	3715	Clear	Clear	Clear	Clear	Clear	Clear
	30	464	445	1176	1245	1534	1596	2403	2521	3508	3384	4480	4665
Control[a]	8	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	387	387	735	890
	30	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	812	890	1160	1044
Control[b]	8	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
	30	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

† Refers to a turbidity value similar to the sterile media control (i.e. the total volume of the assay medium has been inhibited).
‡ Refers to a turbidy value similar to the control indicator culture which had not been seeded with a test organism
a Refers to a control culture medium seeded with L. monocytogenes (NCTC 9863-test organism)
b Refers to a sterile culture control for TSYEA semisolid media.

Certain *Bacillus* spp. (namely, test strain numbers 35, 58, 132, 133, and 170) demonstrated a very strong antagonism against both morphological forms of *L. monocytogenes* at this psychrotrophic assay temperature. Indeed, at an indicator inoculum level of $\leq 10^4$ *Listeria* cells ml^{-1}, a total clearing or inhibition of the suspended *Listeria* cells was achieved. This extreme level of inhibition possibly occurred as a result of (as yet) an undefined antagonistic activity exhibited by the

above mentioned *Bacillus* spp., which had been seeded on the agar surface. On observation of Figure 6.5, it can be clearly seen that the volume (mm^3) of *Listeria* growth inhibition is influenced by the inoculum level or concentration of *L. monocytogenes* cells present in the soft agar. Moreover, the greater the concentration of indicator cells (*Listeria*), the smaller the volume of inhibition detected.

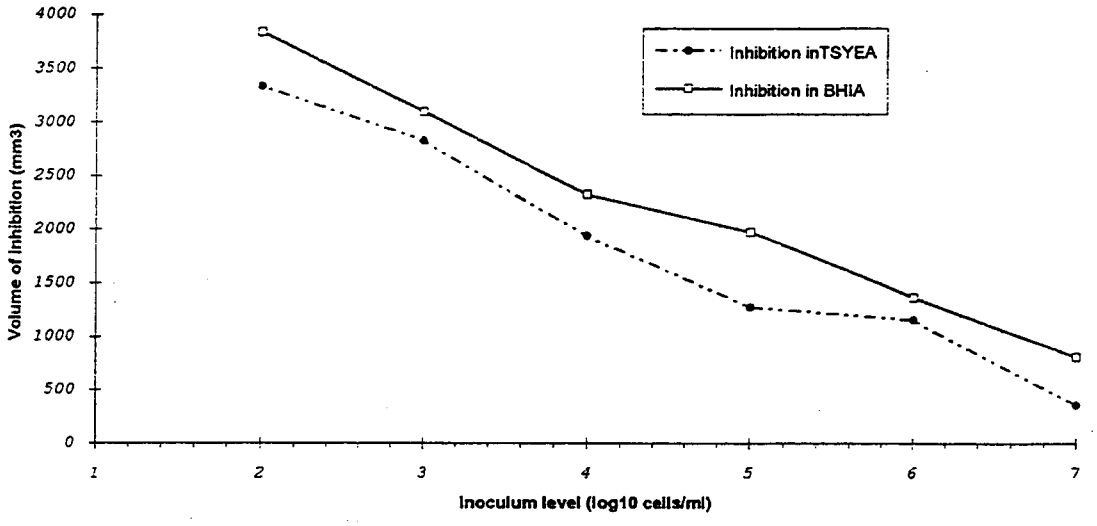


Figure 6.5 Direct antagonistic technique- growth inhibition (mm^3) of *L. monocytogenes* (NCTC 11994) at different inoculum levels in both TSYEA and BHIA semisolid media, as a result of an antagonistic interaction with *B. amyloliquefaciens* (No. 170) at an assay temperature of 30°C.

The variation in *Listeria*'s morphological colony form and/or the type of culture media did not influence the level of inhibition experienced by the indicator cell (at each inoculum level). In order to get a better understanding of the variation in antagonistic activity at 8°C and 30°C assay temperatures (after 6 days storage), a plot of *Listeria* inoculum level (cells ml^{-1}) against volume of inhibition (mm^3) is constructed in Figure 6.6. In this figure the test organism (*B. subtilis*, No.133) has been seeded on the agar surface and allowed to interact with the rough form of *L.*

monocytogenes (NRB2) which had been suspended in TSYEA. Furthermore, complete inhibition of *Listeria* in 10 ml of TSYEA soft agar (designated as "Clear") is equivalent to a volume of 5418 mm^3 (Figure 6.6). It is observed that the lower assay temperature results in enhanced antagonism for this particular combination of test and indicator organism.

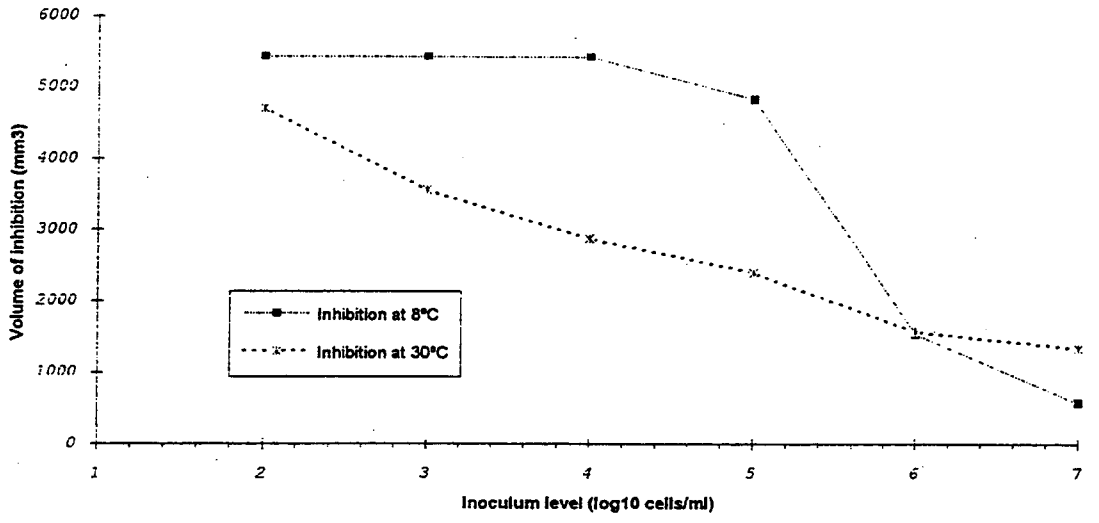


Figure 6.6 Illustrates the effect of inoculum level (*Listeria* cell ml^{-1}) and assay temperature during the 6 day storage, on the inhibition (volume in mm^3) of *L. monocytogenes* (NCTC 11994) by *B. subtilis* (No.133).

The results of this direct antagonistic study suggest that an inoculum of 10^5 *Listeria* cells ml^{-1} (in soft agar) is optimal to the subsequent identification of antagonistic *Bacillus* spp. In addition, psychrotrophic *Bacillus* spp. are capable of inhibiting the development and proliferation of different surface cultures of *L. monocytogenes* at ambient and psychrotrophic (8°C) temperatures. Indeed, the level of antagonism experienced by the indicator organism in soft agar is exacerbated at this near refrigeration temperature (Figure 6.6).

6.1.3 Antagonistic study in liquid media.

After the experiments employing solid and semi-solid media, the growth inhibition of *L. monocytogenes* by *Bacillus* spp. was performed using liquid media (reconstituted infant milk formulae). However, due to the large number of *Bacillus* spp. (i.e. 40 species in total) exhibiting antagonistic interactions against different morphological forms of *L. monocytogenes*, it was not practical or feasible to illustrate every antagonistic interaction. Therefore, one of the test organisms (i.e. *B. mycoides*, No.58) was selected for further antagonistic investigations.

Prior to any antagonistic study, an experiment was carried out to determine the growth profiles of *B. mycoides* and *L. monocytogenes* (NCTC 11994) respectively in tyndallised SMA Gold Cap (an infant milk formula). Both organisms were streaked to single colonies on TSYEA (37°C for 24 hours). An isolated colony of either *B. mycoides* or *L. monocytogenes* was inoculated into duplicate 250 ml Erlenmeyer flasks containing 100 ml SMA Gold Cap and incubated for 24 hours at 30°C under gyrotary cultivation at 110 rpm. After incubation, a decimal dilution of the respective subcultures was carried out in 0.01 M PBS (down to 10^{-5} dilution).

A 1.0 ml aliquot of the respective 10^{-5} dilutions was aseptically transferred into a new set of 250 ml flasks containing tyndallised SMA Gold Cap. The separate test cultures were incubated over a 25 hour period under the same conditions as described above. In order to obtain a total aerobic mesophilic count (CFU ml^{-1}) over the course of the 25 hour storage, samples were aseptically removed at regular intervals, decimally diluted and spread plated onto TSYEA plates (via the spiral and spread plate technique).

The results of this investigation, which are illustrated in Figure 6.7, show that *L. monocytogenes* achieved a greater cell number compared to *B. mycoides* which had been cultivated under the same conditions in infant milk formula.

However, calculation of the respective generation times using the reciprocal of the division rate v (where v= lgN-lgNo/lg2 (t-to)) after only 8 hours at 30°C, illustrates that during the early stage of culture growth *B. mycoides* achieves its cell division (49 mins per division) faster than *L. monocytogenes* (which took 66 mins).

After this preliminary study which compared the growth profiles of *L. monocytogenes* and *B. mycoides* in infant milk formula (SMA Gold Cap) at 30°C, the antagonistic assays were performed. SMA Gold Cap was selected as the culture medium for antagonistic interactions during this investigation, as this growth medium was previously shown to support good development and resuscitation of both *Bacillus* and *Listeria*. The antagonistic assays were performed at either 30°C and/or 4°C (i.e. refrigeration temperature), using the direct and deferred antagonistic methods.

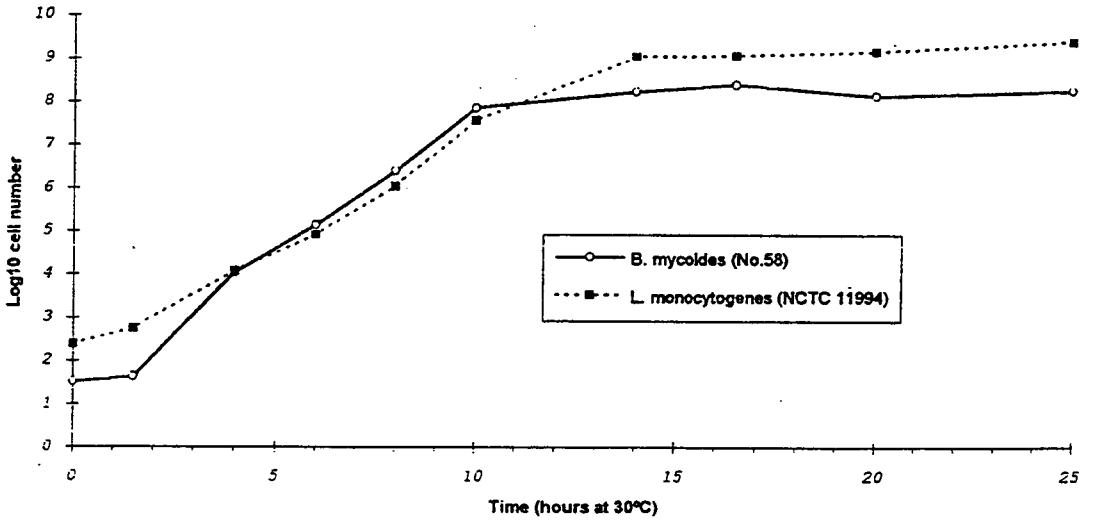


Figure 6.7 Growth of *L. monocytogenes* (NCTC 11994) and *B. mycoides* (No. 58) in SMA Gold Cap at 30°C under gyrotary conditions (110 rpm).

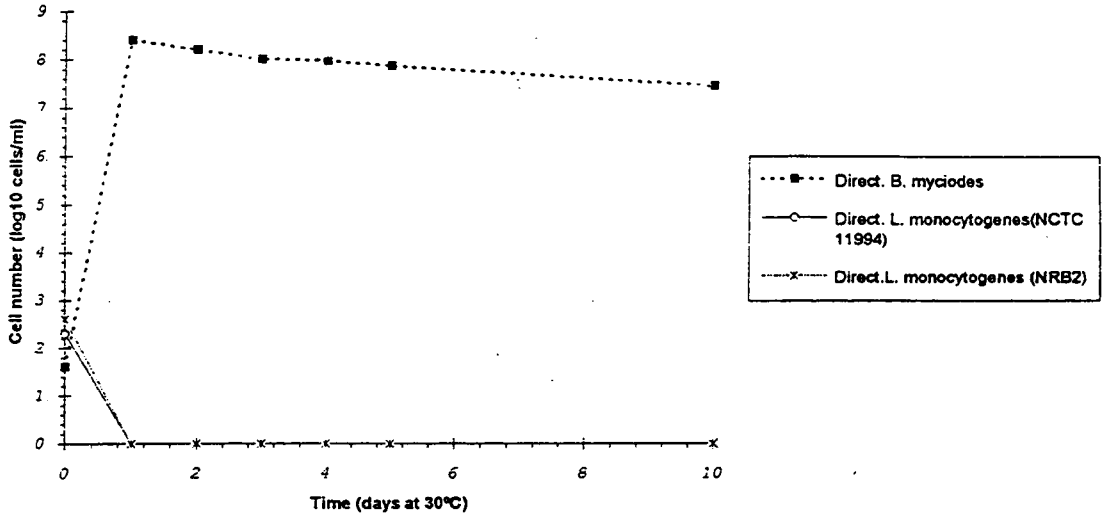
The inoculum for both the test organism (*B. mycoides*) and the indicator organism (*L. monocytogenes*) were prepared in SMA Gold Cap at 30°C as described above. Decimal dilutions of each 20 hour culture were performed in 0.01 M PBS in order to attain a starting cell concentration of approximately 10^2 to 10^3 CFU ml^{-1} in the respective assay media (this applies to both the test and the indicator cultures).

In the direct antagonistic method, both the test and the indicator organisms were inoculated simultaneously into 100 ml of tyndallised SMA Gold Cap (a cell concentration of approximately 10^2 CFU ml^{-1} was achieved for each organism). The deferred method was carried out in two different ways and for this reason they were designated as either deferred method 1 or deferred method 2 (Batista 1993). In deferred method 1, the indicator (*L. monocytogenes*) organism was inoculated first into the 100 ml of IMF and after a 20 to 24 hours incubation period, the test organism (*B. mycoides*) was seeded (again at an initial cell concentration of ~10^2 CFU ml^{-1}). Deferred antagonistic method 2 was simply a reverse of deferred method 1, where *B. mycoides* was inoculated into 100 ml of SMA Gold Cap and incubated for 20 to 24 hours prior to the addition of *L. monocytogenes*.

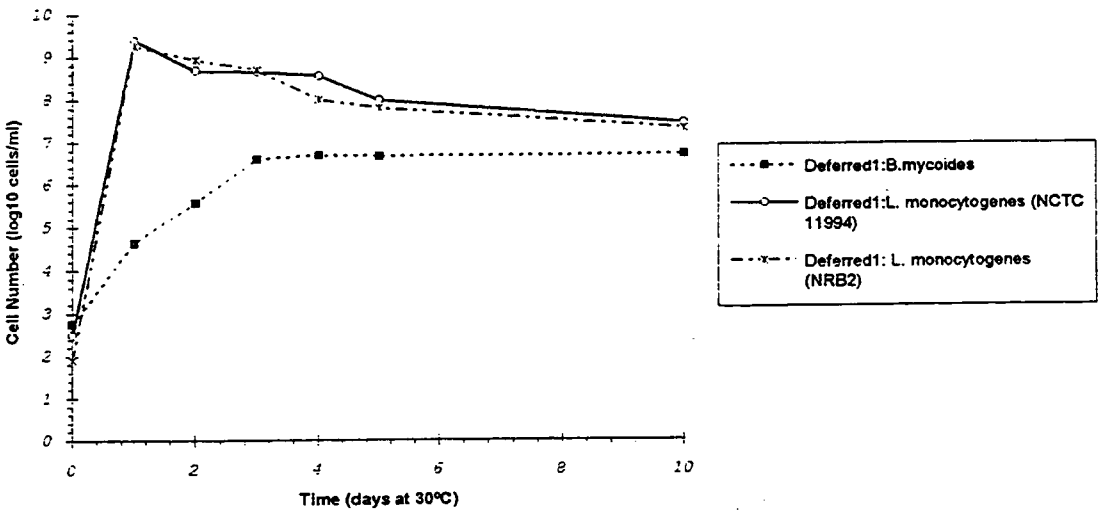
The antagonistic interactions were carried out at either 30°C or 4°C for up to 10 days. Samples were aseptically removed at regular intervals over the course of this observation period and a total aerobic mesophilic counts (CFU ml^{-1}) was performed via the spread and the spiral plate techniques. Indeed, after the first series of samples taken from the direct antagonistic broths (at 30°C), it became evident that the spiral plating technique (section 3.5.6) was not an appropriate surface dilution method as the smaller *Listeria* colonies were overgrown by the larger irregular colonies of *B. mycoides*. For this reason all subsequent dilutions of the assay media were performed by the conventional spread plate technique (section 3.5.3).

The direct antagonistic method (Figure 6.8a) illustrated that when *B. mycoides* and either of the 2 colony forms of *L. monocytogenes* were cultivated simultaneously in infant milk formula at 30°C, *Listeria* cells were not recovered after 24 hours (or 1 day) storage.

(a)


Cell number (log10 cells/ml)
Time (days at 30°C)
Direct. B. myciodes
Direct. L. monocytogenes(NCTC 11994)
Direct.L. monocytogenes (NRB2)

(b)


Cell Number (log10 cells/ml)
Time (days at 30°C)
Deferred1:B.mycoides
Deferred1:L. monocytogenes (NCTC 11994)
Deferred1: L. monocytogenes (NRB2)

(c)

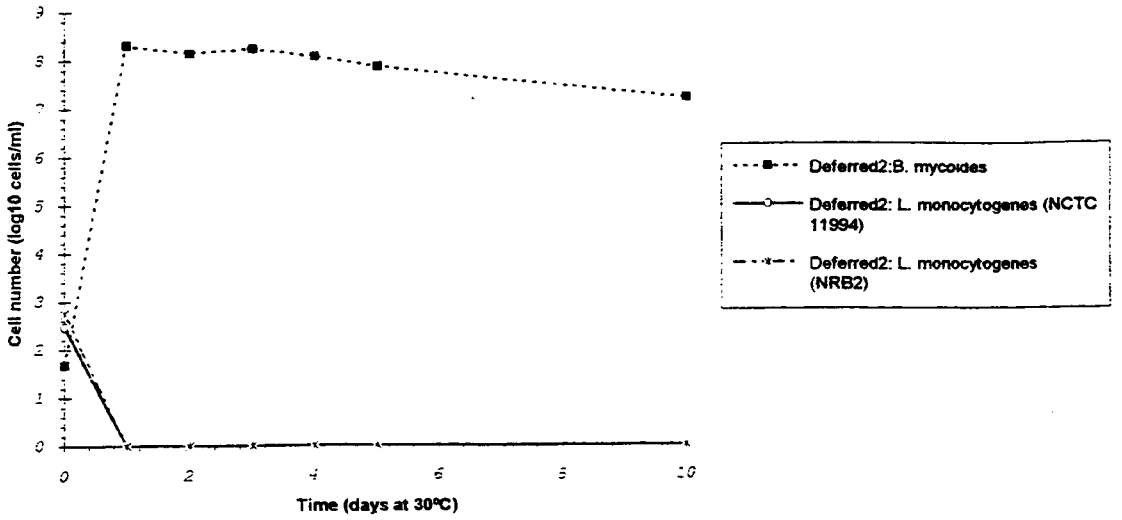


Figure 6.8 Antagonistic interaction between *B. mycoides* (No. 58) and two morphological forms of *L. monocytogenes* (NCTC 11994 and NRB2) in SMA Gold Cap at 30°C: (a) direct antagonistic method, (b) deferred antagonistic method 1, (c) deferred antagonistic method 2.

Meanwhile, the number of *Bacillus* cells decreased steadily over the 10 day observation period. The same level of inhibition was experienced by both colony forms of *L. monocytogenes*. In the experiment in which *L. monocytogenes* was seeded and cultivated a full day before *B. mycoides* (i.e. deferred method 1, Figure 6.8b), the number of *Listeria* cells recovered was high after 24 hours cultivation, however a gradual reduction in cell number was evident over the remaining 9 days incubation. At the same time it was observed that the number of *Bacillus* cells increased steadily to a point at 4 days where the cell concentration remained constant. The *Bacillus* cell population did not exceed that of *Listeria* over the course of this interaction.

When the *Listeria* cells (i.e. from both morphological colony forms) were inoculated the day after *Bacillus* (Deferred method 2 -Figure 6.8c), no *Listeria* cells were detected, while the number of *B. mycoides* cells increased steadily before decreasing

over the remainder of the 10 day observation period. The variation in colony morphology did not influence the level of antagonism experienced by the *Listeria* cells via the direct and deferred antagonistic methods.

The results from the direct and deferred method 2 indicated that *Listeria* cells were strongly inhibited to the point of total exclusion, when cultivated simultaneously with *B. mycoides* in infant milk formula. However, rough and smooth morphological forms of *Listeria* remained detectable when they were cultivated 24 hours prior to the addition of the test organism (i.e. deferred method 1).

When the antagonistic assays were performed at the refrigeration temperature of 4°C, it was observed that, both *Listeria* and *Bacillus*, when inoculated simultaneously via the Direct method (Figure 6.7a) decreased in number over the 10 day observation period. As a similar trend emerged on application of deferred methods 1 and 2, no figures are illustrated to this effect (i.e. where a gradual reduction in microbial cell number was observed for the two organisms in the infant milk formula at 4°C over the 10 day incubation period). The combination of storage period (10 days), assay temperature (4°C) and/or organism was unsuitable to the subsequent development of either *B. mycoides* and/or *L. monocytogenes* (Figure 6.9) Therefore, in order to observe an antagonistic interaction in reconstituted infant milk formula under psychrotrophic conditions, it would be advisable to either increase the period of observation or elevate the assay temperature (>4°C).

Due to the time scale of the overall investigation, in addition to the fact that most parents would not store reconstituted IMF in the refrigerator for excessively long periods, this line of study was not pursued. However, it can be said that storage of reconstituted infant milk formulae at 4°C for up to 10 days (where the initial cell concentrations is less than 10^3 CFU ml^{-1}) is unfavourable to the subsequent development of these particular test organisms.

The antagonistic interaction between *B. mycoides* (No. 58) and *L. monocytogenes* (NCTC 11994 and/or NRB2) in BHI broth (i.e. liquid medium), was also investigated in the absence of living *Bacillus* cells. Due to the difficulty in separating the microbial cells from the nutritional components of infant milk formula, BHI broth was employed as the test liquid medium (Figure 6.10).

The assay was performed in duplicate by inoculating a fixed *Listeria* cell inoculum into 50 ml of filter sterilised *B. mycoides* broth culture (see section 3.14.2.2), in order to achieve a starting cell concentration of approximately 10^2 cells ml^{-1}. The broths were incubated for 24 hours at 30°C , at which point a total aerobic mesophilic count (CFU ml^{-1}) was carried out on TSYEA and LSA plating media. The sterility of the filtered *Bacillus* broth culture was confirmed by performing a total aerobic mesophilic count (CFU ml^{-1}) on the above mentioned plating media.

The results of this experiment illustrated a greater reduction in *Listeria* cell numbers was achieved in the overnight *Bacillus* filtrate broth compared to the *Listeria* control broth. The variation in *Listeria's* colony morphology did not influence the level of antagonism experienced.

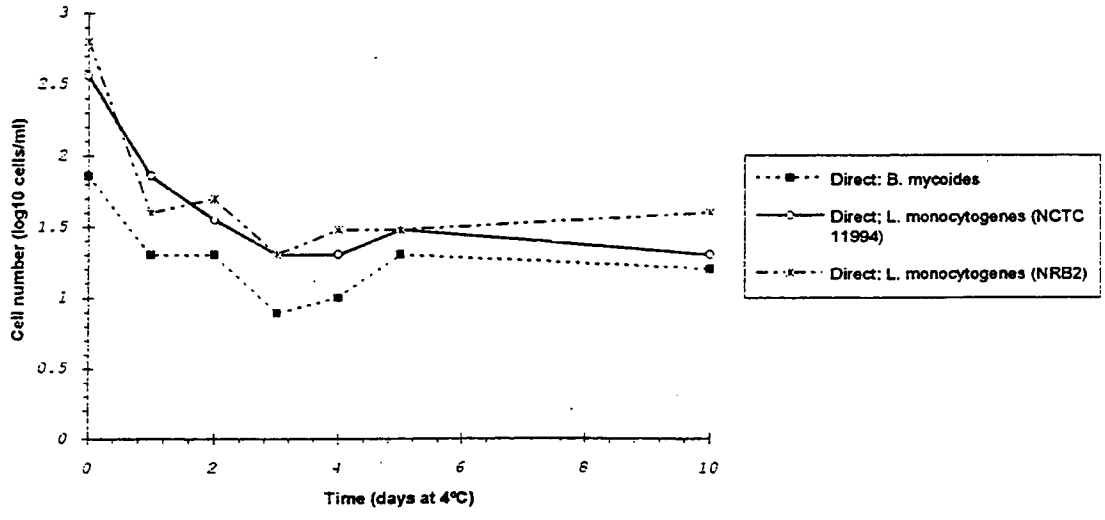


Figure 6.9 Direct method: antagonistic interaction between *B. mycoides* and a rough and smooth morphological form of *L. monocytogenes* in SMA Gold Cap at 4°C.

6.1.4 General conclusion

The results of the experiments studied in this section illustrated that 40 of the 178 *Bacillus* spp. (i.e. 22.5%) originally isolated from reconstituted infant milk formulae exerted varying degrees of inhibition against the growth of smooth and rough colony forms of *L. monocytogenes*.

An initial screen of the large *Bacillus* IMF culture collection for possible *Listeria* antagonists was performed by the direct (stab inoculation) antagonistic method, as recommended by Batista (1993)., where a non selective agar medium was seeded with approximately 10^5 *Listeria* cells ml^{-1} prior to the stab inoculation of the test organism (*Bacillus* spp).

The experimental design of the proceeding experiments confirmed that the combination of indicator inoculum level (10^5 *Listeria* cells ml^{-1}), assay medium (TSYEA), the temperature of interaction (30°C) and the method of antagonism (direct via stab inoculation onto a solid medium) was adequate for the subsequent identification of potentially antagonistic *Bacillus spp.* (which had been isolated from infant milk formulae).

This inhibition was observed, under certain conditions, in all the different assay methods tested. Indeed, in the antagonistic assay employing solid media (agar plates), the direct method appeared to be more accurate and reproducible compared to the deferred methods.

Further investigations using the 40 *Bacillus* spp. (short listed as organisms capable of exerting a strong inhibitory effect on both colony forms of *L. monocytogenes)*, confirmed that the greatest inhibition was observed in TSYEA solid or semi-solid media which had been initially seeded with approximately 10^5 cells ml^{-1}. Inhibition in solid and/or semi-solid media was demonstrated by the appearance of a zone (mm) of clearing around *Bacillus* on the agar surface and a volume of clearance (mm^3) below the soft agar surface respectively. The inhibition process exhibited in BHI agar and Blood agar were inferior to that achieved in TSYEA.

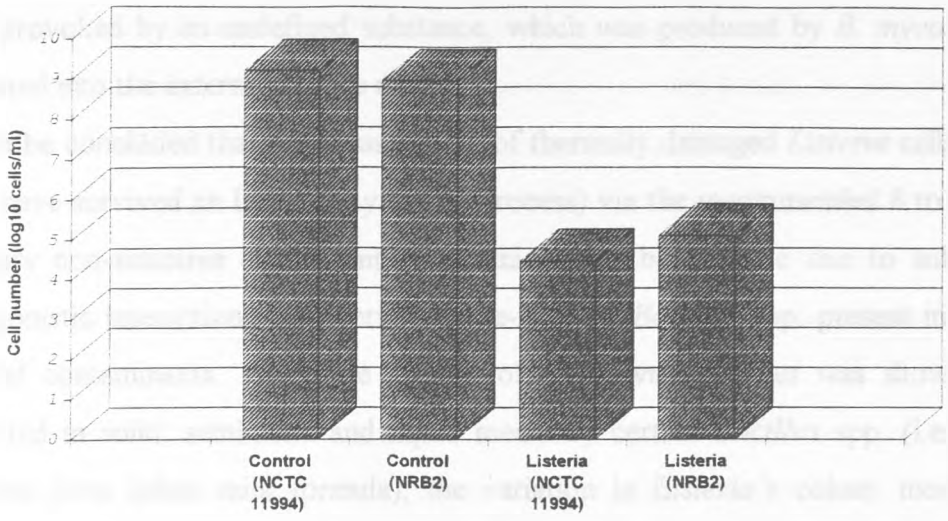


Figure 6.10 Growth inhibition of *L. monocytogenes* NCTC 11994 and NRB2 in a cell free BHI broth culture at 30°C, which had been previously occupied by a 24 hour culture of *B. mycoides*.

By employing 8 psychrotrophic and/or antagonistic *Bacillus* spp. , confirmation that the inhibition process could be performed in semi-solid media was achieved. Moreover, it was demonstrated that a greater level of *Listeria* inhibition occurred at 8°C after 6 days compared to the same antagonistic interactions performed at the higher assay temperature of 30°C.

Further studies carried out in liquid media (i.e. reconstituted infant milk formula) demonstrated that *B. mycoides* exhibited an inhibitory activity against both morphological forms of *Listeria* at an assay temperature of 30°C. Indeed, *B. mycoides* grew to the exclusion of *Listeria*, where *Bacillus* was inoculated either simultaneously or one day before the addition of *Listeria* cells. Experiments carried out in IMF at 4°C were inconclusive, as a similar reduction in both *Bacillus* and *Listeria* cell numbers occurred over the 10 hour observation period.

Investigating the antagonistic process in the absence of living *B. mycoides* cells, it was noticed that a greater inhibition of *Listeria* occurred in the overnight *Bacillus* broth

filtrate compared to the growth of both morphological forms in sterile broths. It is postulated that the inhibitory process observed in the cell free *Bacillus* broth culture was provoked by an undefined substance, which was produced by *B. mycoides* and excreted into the external culture media.

It can be concluded that the resuscitation of thermally damaged *Listeria* cells (which may have survived an IMF spray drying process) via the recommended 8 to 10 hour primary non-selective enrichment stage, might not be possible due to subsequent antagonistic interactions with certain spore-forming *Bacillus* spp. present in IMF as natural contaminants. While the growth of *L. monocytogenes* was shown to be inhibited in solid, semi-solid and liquid media by certain *Bacillus* spp. (i.e. natural isolates from infant milk formula), the variation in *Listeria*'s colony morphology which had been designated as smooth and rough, did not influence the level of antagonism experienced by this bacterium.

A further series of experiments were designed in order to investigate the antagonistic activity of *Bacillus* spp. against other microorganisms. These experiments will be presented in the section 6.2.

6.2 Antagonistic interaction between *Bacillus spp.* and other microorganisms.

In the previous section it was demonstrated that 22.5% of the 178 *Bacillus spp.* originally isolated from infant milk formula inhibited the growth of *Listeria monocytogenes*. The aim of this series of experiments was to establish whether or not these *Bacillus spp.* have the ability to produce substances inhibitory to other Gram positive or Gram negative bacteria and/or to investigate whether this antagonistic activity is specific against certain strains of *L. monocytogenes*.

While *L. monocytogenes* is a known opportunist human pathogen, other species in the *Listeria* genus are considered to be important animal pathogens. The experiment was therefore designed to discover whether the 40 known antagonistic *Bacillus spp.* could inhibit the growth of *Listeria* species other than *L. monocytogenes*. For this purpose, pure strains of *L. innocua* (NCTC 11289), *L. ivanovii* (NCTC 11846), *L. seeligeri*

(NCTC 11856), *L. grayii* (NCTC 10815), *L. welshimeri* (NCTC 11857), *L. murrayi* (10812) and another rough strain of *L. monocytogenes* (NRB5) were tested against the 40 *Bacillus species*.

Indeed, considering that infant milk formulae was implicated as the vehicle of infection (Table 2.6) in a number of *Salmonella* foodborne illness outbreaks (in addition to the on going search for new techniques to control spoilage and potential foodborne pathogens), it was decided to challenge the ability of the *Bacillus spp.* to inhibit the development of *Salmonella enteritidis* (NCTC 8515) and *Escherichia coli* (NCTC 8623).

The antagonistic interactions between the 40 individual *Bacillus* spp. and the other microorganisms were performed using the optimised direct (stab inoculation) antagonistic technique on a solid agar medium (i.e. TSYEA) as described in the previous section (also see section 6.1.1).

The antagonistic assays were performed using the 40 individual *Bacillus* spp. as the test organisms, while the other organisms (*Listeria species, E. coli* and *S. enteriditis*) were designated as the indicator organisms. The test and indicator organisms were streaked to single colonies on TSYEA and incubated at 35°C for 24 hours. The indicator organisms were cultivated under gyrotary conditions at 110 rpm for 20 hours in TSYEB at 35°C to a final cell population level of approximately 10^9 cells ml^{-1}.

Tenfold dilutions (down to the 10^{-4} dilution) of these overnight cultures were then carried out. A 0.1 ml aliquot of this 20 hour 10^{-4} dilution was aseptically inoculated into a fresh set of TSYEB and incubated at under the same set of conditions to ensure a homogeneous distribution of cells. A tenfold serial dilution(down to 10^{-3}) was carried out on each indicator culture and a 0.1 ml aliquot of this 10^{-3} dilution was spread across the surface of a TSYEA plate. A total aerobic mesophilic count (CFU ml^{-1}) was carried out on TSYEA and LSA plates to confirm the indicator (*Listeria* species, *E. coli* and/or *S. enteriditis*) inoculation level were approximately equal in cell number.

Immediately after this, the test organisms (i.e. the 40 strongly antagonistic *Bacillus* spp.) were inoculated by stab into the agar plates which had been seeded with the

indicator strains. The assay plates were then incubated for 24 hours at 30°C, after which the plates were observed for the presence of a clear zone of inhibition around the *Bacillus* colonies. The results of the antagonistic assay are illustrated in Table 6.7.

Table 6.7 Growth inhibition of various microorganisms (mm) by 40 different *Bacillus* spp using the direct antagonistic assay on TSYEA at 30°C.

Test Organism	Stock Number	Indicator Organims											
		A	B	C	D	E	F	G	H	I	J	K	L
B. mycoides	1	NZD	1	2	1.1	0.3	NZD	NZD	1.5	NZD	NZD	NZD	NZD
B. cereus II	3	1.1	1.3	1.5	2	2.5	NZD	NZD	1.4	0.9	0.5	NZD	NZD
B. mycoides	7	3.9	3.4	2.8	3	2.1	1.9	2.6	2.9	1.6	1.1	NZD	NZD
B. cereus II	8	3	3	2.1	3.6	3	3	1.5	3.5	1.6	1.6	NZD	NZD
B. cereus II	15	1.2	1.2	1.9	1.4	NZD	NZD	NZD	2	NZD	NZD	NZD	NZD
B. cereus I	21	0.5	0.5	NZD	1	NZD	1.1	NZD	NZD	NZD	NZD	NZD	NZD
B. subtilis	23	3	3	3.1	4.3	4.5	3.5	1.4	3.5	3.1	3	NZD	NZD
B. cereus I	24	3.8	4	3.6	4.5	3	3.6	1.6	3.7	0.5	2.1	NZD	NZD
B. cereus II	27	4	3.7	3.7	3	2.2	1.5	3.5	2.1	3.1	3	NZD	NZD
B. cereus II	28	3.6	3.6	3.2	2.8	1	1.3	3.1	NZD	3.5	2.1	NZD	NZD
B. cereus II	30	3.1	2.6	1	3.5	1.5	NZD	0.5	1.6	0.3	2	NZD	NZD
B. cereus I	31	0.5	0.6	0.4	1	NZD	NZD	NZD	NZD	0.5	NZD	NZD	NZD
B. megaterium	35	3.5	3.3	3.6	3	2.5	NZD	2.3	3.5	3.4	1.6	NZD	NZD
B. cereus II	38	5	3.9	4.5	4	1.5	3.1	3	4.1	3	1.5	NZD	NZD
B. cereus I	44	NZD	NZD	1	NZD	1.2	NZD	3.1	NZD	0.3	NZD	NZD	NZD
B. cereus I	45	NZD	NZD	1	NZD	NZD	NZD	NZD	0.9	NZD	NZD	NZD	NZD
B. cereus II	48	1.5	1.7	1.5	NZD	3	1.3	4	1.9	0.3	NZD	NZD	NZD
B. mycoides	58	4.1	4.1	3.9	3.5	3.5	3.3	4.1	4.1	0.6	1.6	NZD	NZD
B. cereus II	59	4.6	4.6	4	3.3	3.5	4.1	3.1	3.5	1.6	1	NZD	NZD
B. cereus II	62	0.5	0.5	0.5	0.3	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD
B. cereus I	63	1.8	1.8	2.1	2.5	1.8	NZD	NZD	0.6	NZD	NZD	NZD	NZD
B. cereus I	65	3.1	1.9	5.6	4	2.9	3	1.5	3.4	2	1.5	NZD	NZD
B. cereus II	70	2	1.6	2	1.5	3	NZD	0.5	1.1	1.1	0.9	NZD	NZD
B. cereus II	92	3.1	3	3.8	3.4	3.5	1.5	2	3.8	3	1.4	NZD	NZD
B. cereus II	93	3.5	3.1	3.5	3.5	4	3.5	3.5	3.5	1.6	1.9	NZD	NZD
B. cereus II	99	2.3	1.8	2.2	1	NZD	2	1.3	1.8	3.1	2.1	NZD	NZD
B. subtilis	104	1	1	NZD	0.9	NZD	NZD	1	NZD	NZD	NZD	NZD	NZD
B. cereus I	107	1.3	1.6	2	NZD	0.5	2.5	2	1.6	0.5	1	NZD	NZD
B. cereus I	108	0.3	0.5	1.6	NZD	1.3	0.8	NZD	NZD	NZD	NZD	NZD	NZD
B. cereus II	113	3.5	3.3	3.5	3	3.5	3.1	1.3	NZD	NZD	NZD	NZD	NZD
B. amyl oliquefaciens	118	2	0.5	1.5	3.5	4.1	NZD	NZD	NZD	NZD	1.6	NZD	NZD
B. subtilis	132	3.2	3.6	3.5	4	3.6	1.6	0.9	3.2	4	2.1	NZD	NZD
B. subtilis	133	3.8	3.5	4.5	3.3	1.6	2.5	0.6	3.5	2.1	3.1	NZD	NZD
B. mycoides	148	2.5	1.9	0.5	NZD	1.6	NZD	2.5	NZD	NZD	NZD	NZD	NZD
B. licheniformis	152	2.7	3	NZD	1	1	NZD	NZD	NZD	NZD	NZD	NZD	NZD
B. licheniformis	167	2.4	2.4	2.6	3	3	3.5	0.3	2.1	2.5	1	NZD	NZD
B. megaterium	168	1.6	1.1	1.2	3	3.1	NZD	1.3	0.9	0.9	NZD	NZD	NZD
B. amyl oliquefaciens	170	4.2	3.3	4	2.5	2.5	3.4	1.6	4	1.1	1.1	NZD	NZD
B. subtilis	173	3.1	3.5	3	NZD	3	1.6	1.9	NZD	1.1	NZD	NZD	NZD
L. monocytogenes (11994)	Control	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD
L. monocytogenes (9863)	Control	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD
L. monocytogenes (NRB2)	Control	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD	NZD

A L. monocytogenes (NCTC 11994); *E L. monocytogenes (NCTC 10357);* *I L. innocua (NCTC 11289)*
B L. monocytogenes (NCTC 9863); *F L. murrayi (NCTC 10812);* *J E. coli (NCTC 8623)*
C L. monocytogenes (NRB2); *G L. grayii (NCTC 11815);* *K S. enteritidis (NCTC 8515)*
D L. seeligeri (NCTC 11854); *H L. welshimeri (NCTC 10857)*

It is apparent from the results obtained in Table 6.7 , that the various test organisms (*Bacillus* spp), exhibited different levels of antagonism against the individual members of the *Listeria* genus. Moreover, all the *Listeria* species employed in this investigation experienced varying degrees of growth inhibition, as a result of co-habitating the same culture or assay medium with 15 of the 40 *Bacillus* spp. used in this study. Indeed, the 40 test organisms did elicit an antagonistic interaction (or inhibited the growth) of at least 2 or more *Listeria* spp.

It would appear that these antagonistic spore forming bacteria do not specifically inhibit certain strains of *L. monocytogenes*, but rather they demonstrate varying levels of inhibitory activity against different species in the *Listeria* genus. It was further shown that the other surface culture form of *L. monocytogenes* (NRB2) was also successfully inhibited by the majority of test organisms.

However, the 40 *Bacillus* spp. did not inhibit the development of either *Escherichia coli* (NCTC 8623) or (*Salmonella enteritidis* (NCTC 8515) under the present set of antagonistic conditions. The results of this section suggest that certain *Bacillus* spp. commonly found in infant milk formula, have the potential to inhibit the growth and proliferation of different members of the *Listeria* genus which may have entered the IMF product as a result of (a) a previous bovine infection, (b) contamination at the dairy and/or during the spray drying process, (c) post process contamination.

6.3 Nature of the antagonistic activity produced by *Bacillus* spp.

The development and proliferation of micro-organisms in a culture medium will depend on the provision of a suitable environment. The growth of microorganism may be inhibited by a variety of factors such as, certain physicochemical changes and/or the exhaustion of essential nutrients in the culture medium.

Indeed, the growth of *L. monocytogenes* has previously been shown to be inhibited by a variety of antimicrobial agents produced by the lactic acid bacteria, namely; organic acids, hydrogen peroxide and lactoperoxidase (Spelhaug and Harlander 1989). Another class of inhibitory agents produced by these antagonists are bacteriocins. The criteria mainly used to define bacteriocins are: a narrow spectrum of

activity, a bactericidal mode of action, and a proteinaceous nature (DeMan et al 1960; Tagg et al 1976; Jack et al 1995). Moreover, bacteriocins produced by Gram positive bacteria, may exhibit bacteriocidal activities that are restricted to those spp. which are either closely related and/or which compete for the same ecological niche, or they may be effective against a wide spectrum of Gram positive bacteria including spoilage and pathogenic microorganisms.

The aim of this series of experiments was to endeavour to elucidate the nature of the antagonistic activity produced by *Bacillus* spp. against smooth and rough forms of *L. monocytogenes*. The investigation focused on establishing whether the inhibition was due to the action of acid production, hydrogen peroxide, exhaustion of nutrients, lytic bacteriophages and/or a substance of proteinaceous nature (enzymes, bacteriocins or other antibiotics).

Examination of Table 6.7 reveals that the antagonistic activity produced by *Bacillus spp.* was restricted to Gram positive organisms, suggesting that the inhibition process may have been due to action of a specific compound or compounds rather than the result of a broader bacteriocidal activity (e.g. acid production). Consequently, one of the aims of this part of the project was to treat *Bacillus* spp. cultures with proteases to determine whether the inhibition observed was due to a substance of a proteinaceous nature.

The other candidate antimicrobial agents to be investigated in this section included; production of organic acids which lower the pH of the culture medium, accumulation of hydrogen peroxide in the culture medium; exhaustion of essential nutrients due to co-habitation and the production of a bacteriophage.

Due to the work load involved, it was not practical or feasible to elucidate the nature of the antimicrobial substance produced by all 40 *Bacillus* spp. Therefore, 4 test organisms (i.e. different members of the genus *Bacillus*) were selected from the above 40 *Bacillus* spp., which demonstrated a strong antagonistic activity against all the *Listeria* spp. in the previous section. These test organisms included *B. cereus I* (No.24), *B. mycoides* (No. 58), *B. cereus II* (No. 59) and *B. amyloliquefaciens* (No. 170), whereas the indicator organisms consisted of *L. monocytogenes* (NCTC 11994) and the rough form (NRB2).

6.3.1 Assay to determine whether the inhibitory substance was of a proteinaceous nature.

In order to identify whether the antagonistic activity of *B. cereus I* (No. 24), *B. mycoides* (No. 58), *B. cereus II* (No. 59) and *B. amyloliquefaciens* (No. 170) was caused by enzymes, a bacteriocin or other substance of a proteinaceous nature, the 4 test organism broth cultures were treated with three proteases namely: pronase E (Sigma), trypsin (Sigma) and alpha-chymotrypsin (Sigma), according to the procedures described in section 3.14.6. After the protease-cell treatment, the antagonistic activity of the 4 test organism were tested against smooth (NCTC 11994) and rough (NRB2) colony forms of *L. monocytogenes*.

The experiment was carried out using the direct antagonism method (Spelhaug and Harlander 1989 and Batista 1993). The indicator organisms were cultured separately in TSYEB for 20 hours at 35°C under gyrotary conditions (110 rpm). A 0.1 ml aliquot of the respective 20 hour indicator cultures (using the 10^{-3} dilution) was separately spread over the surface of a TSYEA plate which had been previously treated with 200 µl of the protease (see section 3.14.6). After this, 5 µl of each protease treated *Bacillus* cell suspension was spotted at 3 places (at a distance of 3 cm apart) onto the surface of the protease-treated (*Listeria* seeded) TSYEA agar plates. The plates were incubated for an additional 18 hours at 30°C and observed for the presence of a halo.

The non appearance of a zone of clearing around the *Bacillus* spp. was an indication that the test organism failed to inhibit the growth of the indicator organism (*Listeria*) and that the protease treatment successfully disrupted the bactericidal activity of the proteinaceous substance. Assay plates not treated with the respective protease were employed as controls.

Table 6.8 Sensitivity of the inhibitory activity (produced under normal conditions by the test organisms) to proteolytic enzymes with a rough (NRB2) and smooth (NCTC 11994) colony form of *L. monocytogenes* as the indicator organisms.

Test Organism	Stock No.	Sensitivity of inhibitory activity to proteolytic enzymes with *L. monocytogenes* as the indicator organism[a]					
		α-Chymotrypsin		Trypsin		Pronase E	
		Smooth	Rough	Smooth	Rough	Smooth	Smooth
B. cereus I	24	+	+	+	+	+	+
B. mycoides	58	+	+	-	-	+	+
B. cereus II	59	+	+	+	+	+	+
B. amyloliquefaciens	170	+	+	-	-	-	OG

[a] + sensitivity, - no sensitivity, OG assay plates over grown with the test organism

The antagonistic activity of *B. cereus I* and *II* (No. 24 and 59 respectively) against both morphological forms of *L. monocytogenes* was not observed in the assay plates which had been treated with all three proteases compared to the non- protease treated TSYEA plate controls (Table 6.8). The inhibition process produced by *B. mycoides* (No. 58) against the smooth and rough indicator cultures, was not seen in plates which had been treated with the protease trypsin, however the other two proteases successfully prevented the appearance on zones of clearing (which was observed around the *Bacillus spp.* on the control plates).

The antagonism normally exhibited against *Listeria* spp by *B. amylioliquefaciens* was not observed on the plates which had been treated with alpha-chymotrypsin. This information would strongly suggest that the antagonism experienced during the growth of *Listeria* spp. may be at least partially due to the simultaneous secretion of a proteinaceous substance which has a bacteriocidal effect on the growing *Listeria* cells.

6.3.2 Investigation for the production of lytic bacteriophage

The test for the presence of lytic bacteriophage was carried out according to the method of Lewus et al (1991) as per section 3.14.4. A deferred antagonistic assay was

initially carried out where the test organism was initially stab inoculated onto the surface of a TSYEA plate and incubated at 30°C for 24 hours. Afterward, a TSYEA soft medium containing approximately 10^5 *Listeria* cells ml^{-1} was carefully overlaid across the overnight test organism. The assay plates were incubated for an additional 18 hours at 30°C in order to obtain the zone of inhibition around the *Bacillus spp* (see section 6.14.5).

To detect the presence of lytic bacteriophage, a portion of the clearing zone was cut from the stab deferred assay plate. The agar plug was then added to 3ml of BHI broth and macerated with a sterile glass rod. The mixture was then held at room temperature for 1 hour. A 100 µl amount of the suspension and 100 µl of an indicator organism (grown overnight at 30°C in BHI Broth) were suspended in 4 ml of soft agar (0.7%). The soft agar suspension was aseptically poured over a BHI plate and incubated overnight at 30°C. The retention of plaques would be indicative of phage activity.

Failure to observe any plaque formation on the assay plates (for each of the test organisms) under study, suggested that the inhibition process experienced by *Listeria* was not due to the action of lytic bacteriophages.

6.3.3 Test for hydrogen peroxide production.

The deferred antagonistic test (see section 3.14.3) was used to evaluate and/or confirm that the antagonistic activity demonstrated by *Bacillus* spp. was not a result of hydrogen peroxide production. The test organism was seeded onto the TSYEA assay plate by stab inoculation and allowed to grow for 24 hours at 30°C. The indicator organisms (*Listeria*) were inoculated into 4 ml of soft (0.7%) agar to a final cell concentration of approximately 10^5 cells ml^{-1}) which was to be employed as the overlay culture media. To exclude the possible inhibitory effects due to the action of H_2O_2, catalase (Sigma) was initially added to this overlay agar at a final concentration of 5mg ml^{-1} (Geis et al 1983). The appearance of clear zones around the test microorganism in the presence catalase organism would indicate that the antagonistic activity exhibited by *Bacillus spp*. was not due to hydrogen peroxide production.

Zones of inhibition were identified around the 4 test organisms on catalase containing assay plates seeded with both morphological forms of *L. monocytogenes*, indicating that the inhibition process was not a result of H_2O_2 production.

6.3.4 Test for acid production.

In order to investigate whether the antimicrobial activity of the *Bacillus spp.* might have been due to acid production, the pH of the test culture medium (TSYEB) was measured over a 24 hour period. The test was performed according to the method of Batista (1993), where the 4 test organisms were initially streaked to single colonies on TSYEA plates. An isolated colony of each test organism was inoculated into separate TSYEB culture media. The TSYEB flasks were then incubated at 30°C and the pH was measured using a standard pH meter after 24 hours.

The results showed that the TSYEB non-inoculated test culture control had a pH of 7.0, whereas the pH measurement from the duplicate *B. cereus I* (No.24), *B. cereus II* (No.59), *B. mycoides* (No.58) and *B. amyloliquefaciens* (No.170) TSYEB test cultures was 6.3, 6.3, 6.45 and 6.8 respectively.

Seeliger (1986) stated that the pH range for growth of *L. monocytogenes* is pH 6 to 9, whereas Lovett (1989) went as far as to say that *L. monocytogenes* can tolerate pH fluctuations in laboratory based media down to pH 5.0. On the basis of this information, it would appear that acid production (as related to pH levels at 24 hours) in the test cultures (pH range 6.3 to 6.8) which would not have been solely responsible for the total growth inhibition of *L. monocytogenes*.

6.3.5 Test for the exhaustion of essential nutrients

Considering that the competitive inhibition of a microorganism might be due to the exhaustion of nutrients in the mixed culture medium, an experiment was carried out in order to check this possibility.

A single colony of the test organism (*B. cereus I, B. cereus II, B. mycoides* or *B. amyloliquefaciens*) and the indicator organism (*L. monocytogenes* NCTC 11994)

were inoculated into separate TSYEB subculture media, which were subsequently incubated at 30°C for 20 hours under gyrotary conditions (110 rpm). A tenfold dilution of the respective subculture media was carried out, and a starting inoculum of approximately 10^2 to 10^3 *Bacillus* and *Listeria* cells ml^{-1} was achieved in the test TSYEB media. The TSYEB flasks were then incubated at 30°C under orbital conditions for up to 24 hours. A total aerobic mesophilic count (CFU ml^{-1}) was carried out at regular intervals over the 24 hour observation period on the inoculated TSYEB flasks (by the spread plate technique onto TSYEA and LSA plates).

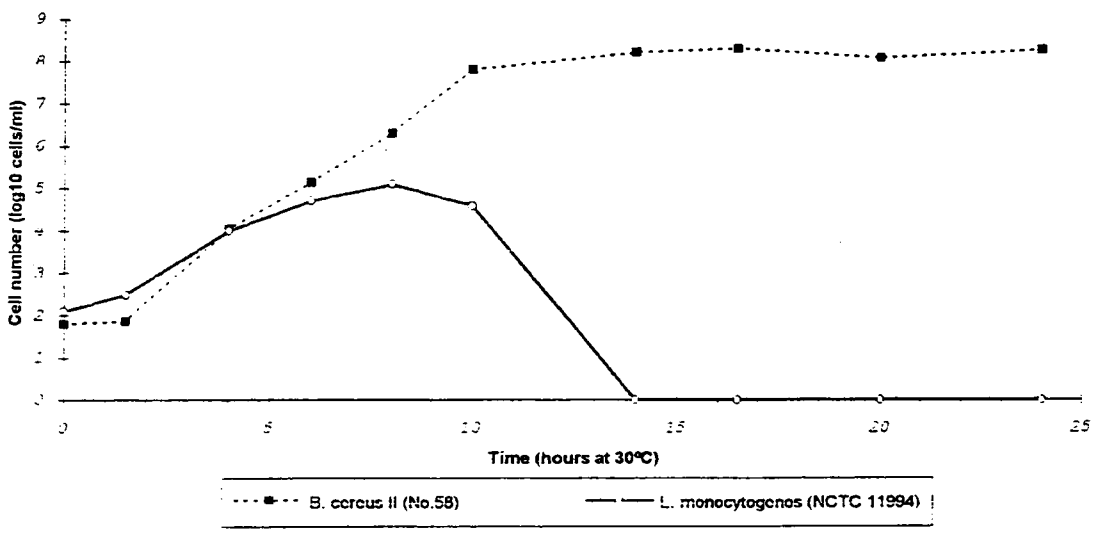


Figure 6.11 Illustrates the antagonistic interaction between *L. monocytogenes* (NCTC 11994) and *B. cereus II* (No.28) in TSYEB at 30°C.

The results of this investigation showed that both the test and the indicator organism grew uniformly to a cell concentration of approximately 10^5 cells ml^{-1} (at approximately 6 hours), at which point the test organism (*Bacillus* spp.) continued to grow to higher cell concentration while the growth of the indicator organism

(*Listeria*) declined. After 14 hours incubation, the test organism grew to the exclusion of the indicator organism (i.e. *Listeria* could not be detected at time samples greater than 14 hours). At *Bacillus* cell concentrations of 10^8 cells ml^{-1} (at 14 hours), the small *L. monocytogenes* colonies could not be detected on the 10^4 assay plates. The same general inhibition process was evident for all 4 test organism. The reduction in *L. monocytogenes* cell number due to the antagonistic activity of *B. cereus II* (No.28) in the TSYEB test culture media is illustrated in Figure 6.11.

Due to the fact that *Bacillus spp.* continued to grow in the TSYEB medium after the growth inhibition of *L. monocytogenes* would suggest that exhaustion of nutrients was not responsible for the reduction and/or elimination of the *Listeria* cells. However, this does not rule out the possibility that the *Bacillus* spp. may have exhausted an essential nutrient required by *L. monocytogenes* for growth.

6.3.6 Conclusion regarding the nature of antagonism

The following conclusions can be made regarding the nature of the antagonistic activity exhibited by *Bacillus spp.* against both culture forms of *L. monocytogenes*.

The results of the protease assays revealed that the inhibition process experienced by *L. monocytogenes* could be eliminated by the addition of certain proteases to the culture media. This would suggest that, the inhibition of growing *Listeria* cells may be at least partially related to the secretion of some bacteriocidal substance of a proteinaceous nature into the culture medium.

The possibility that acid production may have been responsible was excluded, as it was demonstrated that the pH of the culture medium did not go below pH 6.3 after 24 hours and *L. monocytogenes* can tolerate a pH range between 5.0 and 9.0 (Lovett 1989). Furthermore, as the growth inhibition of *L. monocytogenes* was observed on agar plates which had been supplemented with catalase, the possibility of growth inhibition due to hydrogen peroxide production was eliminated.

Similarly, it was shown that the exhaustion of nutrients was not responsible for the antagonistic interaction between the *Bacillus* and the *Listeria* cells, as the former microorganism was able to proliferate in the culture medium after 14 hours indicating

that there was still sufficient nutrients available. The control TSYEB *Listeria* culture achieved a cell concentration of 10^9 *L. monocytogenes* cells ml^{-1} after 14 hours incubation, suggesting that the TSYEB medium is normally suitable for the growth of this microorganism.

The absence of bacteriophage plaques in the TSYEA assay plates indicated that the zones of inhibition were not due to the bacteriocidal action of bacteriophages.

The results of this research suggest that the antagonistic interaction between the 4 *Bacillus* spp. and the 2 pleomorphic colony forms of *L. monocytogenes* may be related to the production and secretion of substances of proteinaceous nature. Furthermore, the variation in colony morphology of *L. monocytogenes* did not influence the level of growth inhibition observed.

7 Discussion

The results obtained in this study will be discussed in 9 sections:

Cultural studies on *Listeria monocytogenes*.
Cultivation, heating and enrichment conditions shown to evaluate the recovery of heat subjected *Listeria* cells.
Identification of improved recovery medium for the isolation of healthy and heat injured *Listeria* cells.
Isolation and identification of *Bacillus* to species level from infant milk formulae.
Microbiology quality of reconstituted infant milk formulae analysed under conditions of preparation and storage abuse.
Diarrhoeal enterotoxin studies.
Infant feeding bottle and/or teat cleaning and sterilization procedures.
Antagonistic interactions.
Nature of the antagonistic activity.

7.1 Cultural studies on *Listeria monocytogenes*.

7.1.1 Identification of test strains.

The typical smooth strains of *L. monocytogenes* were obtained from the National Collection of Typed Cultures (NCTC), while atypical or rough forms were derived from these smooth forms during the course of subsequent thermal inactivation studies. It was considered prudent to carry out morphological and biochemical tests to confirm their identity and to determine a common set of characteristics which would facilitate the identification of wild type strains isolated from food products.
During this study it was found that 2 of the 3 smooth strains gave results which were consistent with morphological and/or biochemical properties typical of *L. monocytogenes*, whereas 1 particular strain (i.e. NCTC 10357) exhibited a negative

CAMP test reaction in both its' smooth and rough morphological form. Indeed, it was observed that repeated CAMP tests employing the same strain resulted in a gradual decrease in the zone of clearing (β-haemolysis) near the *Staphylococcus aureus* line. This reduction in β-haemolytic activity may have been due to the diminished ability of *S. aureus* to elicit an enhanced β-haemolytic reaction on repeated subcultures.

The result of the present study revealed that smooth and rough forms have distinct colonial morphologies. The colonies of the rough form were consistently larger, exhibiting a granular surface, an unbonate to umbilligate elevation and the outer margin varying form slight undulation to marked irregularity. While the smooth form was smaller, with a smooth granulated surface, dew dropped elevation and an entire edge. Indeed, morphological studies performed in this laboratory using the image analyser revealed that the smooth colony form significantly differed in area, perimeter, ferret diameter (i.e. mean diameter of colonies taken from multiple measurements), length/breath ratio and area/perimeter ratio compared to that of the smooth form grown on both non-selective and selective plating media.

These results agree with the findings of Burn (1935), Webb and Barber (1937), (1939) and Batista (1993) who reported that the rough colonies are slightly larger with a rough or wrinkled, dry and matt surface and unbonate center. According to these authors, the edges tends to spread and may show variation form slight undulation to marked dentation. Apart from the work of Batista (1993), studies of colonial variability in *L. monocytogenes* can be primarily found in the early medical microbiology literature, however no evaluation of the occurrence and importance of this phenomenon and/or thermal resistance capability in infant milk formulae was available from the current literature.

In addition to the colonial characteristics, it was observed in the present study that the cell morphologies of the rough variants differed from the smooth form, where the latter consisted of short rods (most of these being arranged as single or paired cells), while the rough forms consisted predominately of rods arranged in short to long chains (up to 60 μm in length) and/or single and paired rod shaped cells. In the rough type, cell arrangements taken from the centre of the colony consisted of a mixture of single, paired and short to long chains, while samples analysed form the outer edge

were comprised predominantly of long chains. The findings from this study are in agreement with Seeliger and Jones (1986) who described that, in rough cultures, chains of 6-20 μm or more often appear. In fact, Seeliger (1961) reported the occasional occurrence of chains up to 275 μm in length.

Barber (1939) also demonstrated that rough variants of *L. monocytogenes* consisted of long cell chains and that the length of chains decreased with prolonged incubation. The author reported that at 37°C, early cultures (7-12 hours) consisted almost entirely of long chains averaging 60 μm. After 24 hours, chains from 10-30μm predominated and long (4.5-8 μm) and short (1.5-3 μm) rods were also evident. After 48 hours, rods from 1 to 5 μm predominated, short chains from 10-12 μm were present in moderate numbers and there were a few coccal forms (no further breakdown occurred after 48 hours). This author did not provide any explanation as to the reason for this change in cell morphology.

More recently, Jorgensen *et al.* (November 1995) revealed that the cell morphology of *L. monocytogenes* was markedly affected by the osmolarity of the growth medium. Cells grown at 30°C in culture media and minced beef containing 1.5 mol l^{-1} NaCl became up to 50 times longer than cells grown in media with 0.09 mol l^{-1} NaCl. By subjecting the elongated cells to an osmotic down shift (1.5 mol l^{-1} to 0.09 mol l^{-1} NaCl) the authors revealed that the elongated cells formed septa within 2-3 hours and after an additional 5 hours incubation the cells regained their more typical short length. However, no mention was made of any association between the change in cellular morphology (described as 'elongated' cells) and colony morphology. In addition, these authors failed to mention the stability of the elongated cellular form and/or provide an explanation for this sudden significant change in cell morphology.

It has been suggested that a major extracellular protein secreted by *L. monocytogenes*, p60 is correlated to the formation of this long chain form. Kuhn and Goebel (1989) reported that the reduction in the amount of p60 leads to the formation of long cell chains where the individual cells are separated by double septae. However, the addition of partially purified p60 to this rough form disrupts the cell chains resulting in a return to the wild type morphology. The influence of p60 on the morphology of rough variants was also demonstrated by Wuenscher *et al.* (1993), who suggested

that this extracellular protein had murein hydrolase activity which is involved in the septation of daughter cells during cell division. In this present study, the rough forms exhibited morphological and biochemical properties characteristic of *L. monocytogenes*, as described by Seeliger and Jones (1986).

Rough culture forms of *L. monocytogenes* emerged during this study after 24 to 48 hours post heat treatment, where smooth *Listeria* cells were initially cultivated in reconstituted infant milk formulae at temperatures >40°C followed by heat treatment at either 56°C, 60°C or 62.8°C. It is therefore proposed that this change in morphology is the result of either; a reduction or elimination of the p60 protein due to detrimental effect of heating; and/or a reduction (or failure) in the level of *iap* (invasion associated protein) expression, which is the gene encoding the transcription this murien hydrolase; or that this gene is plasmid borne and lost during the thermal inactivation process.

7.1.2 Performance of identification kits.

Comparing the performance of identification kits, it was concluded that the API *Listeria* and MICRO-ID *Listeria* test systems performed equally well, while results form the API-Coryne test kit revealed a poor identification profile to species level. The rough and smooth colony forms exhibited similar profiles in these commercial biochemical/fermentation galleries. The limited identification of the API-Coryne system was also observed by Kerr *et al.* (1993), while demonstrating that this system correctly identified all 103 *Listeria* isolates to the genus level, only 20% were presumptively identified to the species level. Further tests, such as β-haemolysis and the CAMP test were required for the identification to the species level.

Several other authors have compared the efficacy of conventional biochemical and morphological identification methods with that of the commercial biochemical galleries test systems for the rapid identification of members of the genus *Listeria*. Bannerman *et al.* (1992) concluded that the MICRO-ID *Listeria* system supplemented with a CAMP test, correctly identified 409 (98.8%) of 414 *Listeria* stains studied. Moreover, these authors demonstrated that the MICRO-ID *Listeria* test kit

successfully identified 12 other Gram positive non-spore forming rods as not belonging to the genus *Listeria*.

The work of Robinson and Cunningham (1991) agreed with that of previous authors, revealing that the MICRO-ID *Listeria* system was comparable to that of conventional methods, as both techniques correctly identified presumptive *Listeria* organisms to species level. In addition, other Gram positive bacillus tested were not misidentified as *Listeria.*

The findings of this study are in agreement with the work of Bille *et al.* (1992), where these authors showed that the API *Listeria* system correctly identified 85% of 646 *Listeria* isolates at the species and subspecies level without supplimentary tests. However, these authors did not challenge the efficacy of this biochemical test system to identify rough culture variants of *L. monocytogenes*. With the API *Listeria* test system, the CAMP test is replaced by the fermentation of glucose-1-phosphate and the DIM test for *L. ivanovii* identification (avoiding the need to employ the CAMP test).

Results from the present study showed that the rough variants isolated in this laboratory did not significantly differ in either fermentative or biochemical characteristics compared to that of the smooth forms. However, an API 50 CHB sugar fermentation profile analysis of these atypical colonies revealed that they either failed to utilise sugars and/or fermented different sugars compared to that of the parent smooth form. This change in fermentation pattern may be due temporary of permanent damage to the genes (and/or promoter sequences) responsible for the eventual synthesis of these enzymes, or loss of a plasmid which carries the genes coding for the synthesis of these enzymes (which may also contain the gene responsible for the transcription of the p60 protein).

These findings are in accordance with Burn (1935) who described only slight alterations in fermentation ability. The author demonstrated that the fermentation of lactose, glycerol and/or sorbitol in rough cultures (within 24 hours at 37°C) differed from the delayed fermentation observed in the original strains. However, in another variant, the sugar fermentation profile remained unaltered.

Similarly, Hunter *et al.* (1950) reported that only a few of the variant cultures differed from the parent strains in fermentation ability, biochemical reactions and antigenic structure. Indeed, mannitol and galactose fermentation was seen in 2 strains, whereas both sugars were not utilised by the parent smooth strains. These strains (as well as another 4) lost the ability to ferment one or more of the carbohydrates (i.e. arabinose, dextrin, glucose, lactose, melizitose, raffinose, rhamnose, salacin, sucrose and/or xylose) which the parent strains had fermented.

In conclusion, the conventional morphological and biochemical test supplemented with certain commercial biochemical/fermentation test kits (i.e. API *Listeria* and MICRO-ID *Listeria*) successfully identified the smooth and rough strains of *L. monocytogenes* in this study. For the identification of presumptive *Listeria* spp. from contaminated foodstuffs (including IMF), the results of this study recommends the isolation of the organism to purity on both TSYEA and LSA plates, followed by performing: Gram stain reaction, demonstration of tumbling motility, catalase test, Henry oblique illumination and CAMP test which are to be supplemented with an API *Listeria* and/or MICRO-ID *Listeria* reaction profile.

Indeed, cultivation of presumptive *Listeria* cells on LSA (Oxford formulation) plates improves identification as isolates exhibit a black halo surrounding the colony (this results from the hydrolysis of esculin where certain bi-products react with ferric ammonium citrate to produce a black precipitate in the agar medium).

7.2 Cultivation, heating and enrichment conditions shown to enhance the recovery of heat treated *Listeria* cells.

7.2.1 Generation of a homogeneous concentration of *L. monocytogenes* cells.

This study revealed that a fixed concentration of suspended *Listeria* cells could be determined by way of turbidity measurements (at a fixed wavelength of 440 nm) and by Total Aerobic Mesophilic Counts (CFU ml^{-1}). There was a good correlation

between the turbidity measurements at 440 nm and that of the actual cell concentration in the range 10^9 to 10^5 CFU ml^{-1}. Below a Total Aerobic Mesophilic Count of 10^5 CFU ml^{-1}, the spectrophotometer could not be successfully employed to indicate the number of suspended cells present. By adjusting the cell culture after 24 hours growth to approximately 10^9 cell ml^{-1} (i.e. equivalent to 2.0 absorbance units), a starting inoculum of 10^2 cell ml^{-1} could be obtained by carrying out serial dilutions in a phosphate buffer.

The use of turbidity measurements to estimate a suspended *L. monocytogenes* concentration has been employed by other researchers. Bradshaw *et al.* (1991) adjusted the cell density of the final TSYEB culture at 625 nm to an absorbance of 2.0 which corresponded to approximately 10^9 CFU ml^{-1}. While Bunning *et al.* (1986) demonstrated that a cell density of 10^9 CFU ml^{-1} corresponds to an absorbance value of 0.8 at 625 nm in TSYEB.

With the aid of turbidity measurements, supplemented with Total Aerobic Mesophilic Counts (CFU ml^{-1}) on TSYEA and LSA plates, the stages of growth for 3 smooth strains of *L. monocytogenes* (NCTC 11994, 9863 and 10357) were determined in a variety of broths (BHI broth, TSYEB *etc.*) and/or reconstituted infant milk formula (IMF). The results of this present study revealed that these *Listeria* cells achieved a late logarithmic to early stationary growth period of growth at 20 hours cultivation at 37°C.

Indeed, the 3 test strains exhibited a similar semi-logarithmic growth profile in the various culture media, and the study revealed that all strains successfully grew in reconstituted infant milk formulae. Cells which were cultivated in a stationary position (0 rpm) at 37°C, achieved a final cell concentration which was lower by a factor of 10 (i.e. 10^8 CFU ml^{-1}) compared to growth under orbital conditions (110 rpm). This increase in cell number may have been attributed to the homogeneous mixing and distribution of cells during shaking at 110 rpm.

Reconstituted IMF that was sterilised via autoclaving at 121°C was shown to support inferior growth compared the same strains being cultivated in IMF which had been tyndallised to sterility. These standard growth curves were used to obtain a

homogeneous concentration of cells (10-100 cells ml^{-1}) in subsequent heat resistance studies.

7.2.2 Thermal resistance characteristic of *Listeria monocytogenes*.

The results of this present study have shown that the heat resistance of both smooth and rough forms of *L. monocytogenes* can be significantly increased by modification of the cells immediate environment (i.e. certain cultivation and heat treatment) prior to and during thermal inactivation. While the recovery of both healthy and sublethally injured cells was influenced by the dilution, enrichment and isolation conditions. This study agrees with the work of Hansen and Rieman (1963) and Busta (1978), who described how many related factors such as: the time and temperature of exposure; the physiological age and cell density during heating; the composition of the growth medium, heating, diluent, enrichment and plating medium can influence the recovery of heat treated non-spore forming microorganisms.

Initial thermal studies revealed that cells heated to and held at 62.8°C for 30 mins were not recovered by conventional identification methods. Indeed, it was observed that the majority of the suspended cell test culture was eliminated during the warming up period and the early holding stages (at this bactericidal temperature). This is in agreement with the work of Tomlins and Ordal (1979) where these authors described the effect of a heating process as the combined influence of the warming up, holding and cooling stages. Improved recovery of these heat treated cells (which were directly plated without subsequent enrichment) was observed on non-selective plates (i.e. TSYEA). These heat treatment studies revealed that the LSA plating medium supported the growth of uninjured and a very low number (if any) of sublethally damaged *Listeria* cells, while the non-selective TSYEA plating medium allowed the recovery of healthy and a larger number of heat injured cells.

In addition, the incubation of plating media for 72 hours (instead of just 24 hours) resulted in a significant increase in number of heat treated cells recovered. Conversely, the lowest recovery of thermally treated cells was observed on selective plating media (i.e. LSA) that had been incubated for only 24 hours prior to enumeration. Indeed, it

is well established that a greater number of sublethally injured *Listeria* cells can repair their injury and subsequently form colonies on non-selective as opposed to selective plating media which have been supplemented with either antibiotics, metal ions and/or dyes (Buchanan *et al.* 1988, Fedio and Jackson 1989, Linton *et al.* 1990, Schoeni *et al.* 1991 and In't Veld *et al.* 1994).

Listeria cells were heat treated in their stationary phase of growth in this present study. Indeed, Linton *et al.* (1990) revealed that *Listeria* cells heat treated in their stationary phase of growth were more heat tolerant compared to cells which had been treated in their exponential stage of development.

This investigation showed that cultivation of cells at the elevated temperature of 40.2, 41 or 42°C under static conditions (0 rpm) prior to heating significantly improved the recovery of cells, compared to cells which were shaken at 110 rpm at the lower temperature of 37°C. Indeed, cells which were cultivated in either tyndallised whole milk and/or infant milk formulae at 40.2, 41 or 42°C in a stationary position prior to heating were recovered after 10 mins holding at 62.8°C via the direct plating technique. This was a significant improvement, as some cells were not detected after only 2 mins at 62.8°C when they were grown at 37°C under orbital conditions.

While most thermotolerance studies have examined *Listeria* cells grown at or below 37°C (Bearns and Girard 1956, Bradshaw *et al.* 1986, 1988, Donnelly *et al.* 1986, 1987, Golden *et al.* 1988), the normal bovine body temperature is approximately 39°C, but this may rise to as high as 42.8°C as a result of a severe listeriosis infection. The results of these initial heat treatment studies are in agreement with the findings of Knabel *et* al. (1990) and Farber *et al.* (1992), where these authors showed that cultivation of *L. monocytogenes* cells at temperatures at or near maximum for physiological growth (43°C) will enhance their heat resistance.

Indeed, Knabel et al. (1990) revealed that cultivation of *Listeria* cells at 43°C prior to HTST pasteurisation, in addition to enriching these injured cells under strict anaerobic conditions, permitted low level survival. Indeed, these authors illustrated that the decimal reduction time for cells grown at 43°C, heated at 62.8°C and recovered anaerobically was at least 6 fold greater compared to cells grown at 37°C and recovered aerobically. This present study revealed that the enrichment of heat treated

Listeria cells in long, narrow glass bottles (without agitation) enhanced the recovery of healthy and heat injured cells. Enrichment of heat treated cells under these conditions may have resulted in localised anaerobic environment. Knabel *et al.* (1990) suggested that the reason for this increased heat resistance was due to the accumulation of heat shock proteins prior to heat treatment, supplemented with the repair of inactived enzymes (such as catalase and superoxide dismutase) in oxygen sensitive heat stressed cells under anaerobic conditions

Other authors that support this popular theory regarding the synthesis of heat shock proteins (HSP's) which may partially protect the cells against subsequent exposure to normally bactericidal temperatures include,Golden *et al.* 1988, Linton *et al.* 1990, Busch and Donnelly 1992 and Meyer and Donnelly 1992. While Beuchat (1978) speculated that growth at higher temperatures resulted in the production of thermostable membranes, which result in increased thermotolerance.

Other research has shown that certain Gram negative (Elliker and Frazier 1936) and Gram positive bacteria (White 1953, Jenkins *et al.* 1986) cultivated at above normal physiological temperatures exhibit greater thermal resistance.

While growth at above optimal temperatures (>37°C) prior to heat treatment significantly enhanced the thermotolerance of all the test strains in this study, this investigation does not support the work of Fedio and Jackson (1989) who demonstrated that tempered cells at 48°C for 1 hour prior to heat treatment at 60°C enhances their heat resistance. The tempering of *Listeria* cells for 30 mins at 47°C prior to heating at 62.8°C did not influence their recovery in this study. Indeed, Bunning and co-workers (1992) support this finding, as they demonstrated that tempering at 47°C for 15 mins prior to heat treatment did not influence thermal resistance of *L. monocytogenes*.

However, this study agrees with the findings of Knabel and his coworkers (1990), where enrichment of the heated cells compared to simply direct plating, resulted in far greater recovery of these heat injured cells (as they were detected at longer exposure times). Moreover, cells which were cultivated (40.2°C) and enriched in a stationary position (0 rpm) were detected after 25 minutes at 62.8°C in reconstituted infant milk

formulae and whole milk samples (i.e. enriching treated cells in a stationary position compared to agitation improved their recovery).

The cultivation, heat treatment, dilution and enrichment of cells in the same type of test medium (i.e. keeping the composition of the medium constant throughout each stage) enhanced the recovery of injured cells in this investigation. Indeed, the optimum recovery of *treated* cells occurred under conditions where: the cells were cultivated at 40.2°C in a stationary position, heated and diluted in a medium similar in composition to the initial growth medium, enriched in a stationary position for 2-3 days at 30°C and incubated on non-selective plating media for 3 days at 37°C prior to enumeration.

This study revealed a difference in the thermotolerance between strains of *L. monocytogenes*, where the serovar 4b test strains (i.e. NCTC 9863 and 11994) were recovered in greater numbers and after longer exposures at 62.8°C compared to the serovar 1a strain (i.e. NCTC 10357). This finding was consistent for all cultivation conditions, culture media, heat treatment temperatures and plating media employed.

This study supports the findings of a number of reports which suggest that the heat resistance capability of *L. monocytogenes* can vary according to the particular strain employed. Golden *et al.* (1988) illustrated that one strain, Brie 1, was significantly more heat resistant compared to other strains; however, the authors believed that it was unlikely that this strain would survive pasteurising. Subsequent research by Sorquist (1993, 1994) also demonstrated a variation in thermal resistance among 12 serotypes of *L. monocytogenes* at a heat treatment temperature of 62°C. However, these authors did not investigate the possible difference in thermotolerance between serotype 4b strains (main serotypes implicated as aetiological agent in a number of food borne outbreaks of listeriosis) and other serotypes.

During the course of these thermal studies, rough colony variants of *L. monocytogenes* appeared. Conditions shown to favour their development included: cultivation of cells at 40.2°C, thermal inactivation at either 56°C, 60°C or 62.8°C and enumeration on non-selective plating media. Smooth test strains which were either cultivated at 37°C or not subjected to a heat treatment did not exhibit any change in their colonial morphology. These rough forms were revealed to be relatively stable

(reversion rate of 1-3% depending on the strain employed). Cultivation and heating of cells in reconstituted infant milk formulae and/or whole, semi-skimmed and skimmed milk resulted in the emergence of rough forms. Laboratory based media (e.g. TSYEB and BHI broth) did support the development of the rough forms, however they appeared at a lower frequency compared to the milk based test media.

In order to determine the heat resistance capability of both colonial forms, thermal studies were repeated at a variety of heat inactivation temperatures (56, 60 and/or 62.8°C) at various holding time regimes. This study revealed that by taking regular Total Aerobic Mesophilic Counts (CFU ml^{-1}) over shorter holding time periods at 62.8°C and/or employing lower heating temperatures (e.g. 56°C or 60°C) over longer holding times, a measure of the cells thermotolerance was obtained (i.e. via construction of a thermal death rate plot of the log number of cell survivors against time).

However, construction of these thermal death rate plots for the smooth form (cultivated in a variety of media) resulted in a curved death rate, i.e. a shoulder section was observed prior to an accelerating death rate. As *L. monocytogenes* cells did not exhibit a normal linear logarithmic death rate, determination of heat resistance capabilities via D and Z values could not be achieved as these thermal death rate kinetic values can only be calculated from linear death rate plots (Tomlins and Ordal 1979).

However, these thermal death rate curves were successfully straightened by application of the linearisation formula $(\log N_0 - \log N)^a = kt + c$, and a measure of their heat resistance (i.e. the reciprocal of the thermal death rate constant k which is analagous to the logarithmic D value) was calculated. Furthermore, the log of these 1/k values was employed in a similar manner to that of the log D values in order to determine the heat tolerance of each smooth strain.

This linearisation formula was initially developed by King *et al.* (1979) to linearise the survivor curves of heat resistant ascospores of the mould, *Byssochlamys fulva*. The log survivor curves of this mould was also characterised by a shoulder section prior to an accelerating death rate. Tomlins and Ordal (1979) revealed that the shoulder effect can be explained by; the gradual accumulated effects of heating, resulting in injury but

not loss of viability and/or clumping of cells. While a large number of researchers investigating the thermal resistance of *L. monocytogenes* have illustrated a log survivor curve for this organism (i.e. shoulder section prior to an accelerating death rate), all these authors have plotted a best fit straight line through these data points in order to determine the log D values (Donnelly *et al.* 1987, Mackey *et al.* 1990, Knabel *et al.* 1990, Quintavalla and Campaini 1991, Fain *et al.* 1991 and Sorquist 1993)

Results from these controlled thermal death rate plots supported the findings of earlier thermal studies, where: a variation in the heat resistance was identified among the test strains (i.e. the serovar 4b strains being more thermotolerant compared to the 1a strain); cells cultivated at 40.2, 41 or 42°C exhibited greater heat resistance compared to cultivation at 37°C, and employing IMF as the growth, heating menstruum and diluent provided good recovery of heat treated cells.

However, rough forms demonstrated different thermal death rate curves to those of the smooth forms, exhibiting an initial shoulder section in addition to a tail at the longer holding times. Application of the linearisation formulae to this survivor curve did not provide a straight line plot. Therefore, calculation of the thermal death rate constant k was not possible. However, examination of the rough and smooth survivor curves in a variety of test media (for test strains grown, heated and enumerated under the same conditions) consistently showed a tailing effect at the end of the rough form curve, where these cells were being recovered at longer holding times. On the basis of this information it would appear that these atypical colonial forms are more heat tolerant compared to the standard smooth form. This result resembles the correlation between thermotolerance and formation of filamentous cells observed in *Aquaspirillum arcticum*, where long cells were associated with enhanced heat resistance (McCallum and Inniss 1990).

Jorgensen and his co-workers (November 1995) support these findings, where a similar thermal death rate plot (i.e. shoulder and tailing effect) emerged when elongated cells of *L. monocytogenes* were heat treated at 60°C. The authors revealed that osmotic up-shock (cells grown in media with 0.09 mol l^{-1} NaCl before being subjected to a short osmotic up-shock in media containing 0.5,1.0 and 1.5 mol l^{-1}

NaCl) and osmotic adaptation (cells grown in media containing 1.5 mol l^{-1} NaCl) resulted in a 1.3 to 8 fold increase and a 2-3 fold additional increase in heat resistance respectively. While the elongated cells exhibited an enhanced thermal resistance (as illustrated in the thermal death rate plots), Jorgensen *et al.* (1995) failed to find any correlation between thermotolerance and the formation of long cell chainss.

Increased survival under high osmotic conditions has been reported for other foodborne pathogens (Calhoun and Frazier 1966; Tuncan and Martin 1989; Summer *et al.* 1991) and is thought mainly to be related to reduced a_w and also the nature of the solute employed (Gould 1989). Demonstration of both alteration in cell morphology and enhanced thermal resistance for *Listeria* cells grown under conditions of osmotic up-shock and/or osmotic adaptation is very relevant to the present study, as the low a_w of dried infant milk formulae would render surviving non-spore forming cells in a state of osmotic stress (Doyle *et al.* 1985 and Johnson *et al.* 1988).

In conclusion, a set of cultivation, heat treatment, dilution, enrichment and enumeration conditions were identified in this study which enhanced the recovery of heat treated smooth and rough forms of *L. monocytogenes*. It appears that the heat resistance of each test strain was dependent on the combined effect of these cultural, heating and enrichment conditions (as these parameters did not act independently of one another). Both morphological forms did not exhibit a logarithmic thermal death rate plot and only the smooth form survivor curve was successfully linearised for the purpose of calculating thermal resistance characteristics. Despite this, it does appear that the rough strains under study were more heat tolerant than the smooth form due to the tailing effect exhibited by the former organism in the terminal section of its survivor curve.

7.3 Identification of a suitable recovery method for the detection of low numbers of heat *treated Listeria* cells.

The study revealed that factors previously shown to enhance the heat resistance of *Listeria* cells were successfully employed to improve the recovery of thermally treated

cells, isolated from artifically inoculated infant milk formulae. The efficacy of a variety of resuscitation broths were challenged with a small number of heat treated cells which were suspended in PBS. Initial studies revealed that it was better to obtain a low concentration of healthy and sublethally injured cells by cultivation and heat treatment in TSYEB followed by resuspension in the phosphate buffer (PBS), rather than growth in TSYEB followed by resuspension in PBS and then heat treating. The results of Tomlins and Ordal (1979) agreed with the results of this study where organisms suspended and heat treated in a phosphate buffer exhibited lower decimal reduction times compared to being heated in other culture media (e.g. milk, nutrient broth).

However, the lowest recovery of cells occurred when *Listeria* was suspended and heated in PBS prior to enumeration. Cells suspended in phosphate buffer during heating or heat treated in the TSYEB culture medium (followed by resuspension in PBS) were less heat tolerant and therefore recovered in fewer numbers compared to the same cells grown, heated, and enumerated (without any subsequent transfer to PBS) in TSYEB.

Initial enrichment studies showed that resuscitation broths which were not supplemented with either antibiotics or dyes proved to be the most suitable media for the resuscitation of thermally *treated* cells. The addition of glucose and/or yeast extract to these non-selective enrichment broths did not significantly enhance the resuscitation of uninjured/injured cells. While TSYEB, BHI broth and SMA Gold Cap (IMF) were shown to be equally efficient at recovering low numbers of heat treated cells, the FDA and USDA approved primary selective enrichment broths (either with or without their respective antibiotic supplements) consistently gave lower recovery of cells.

A similar result was obtained by Potel and Beuchat (1995) who compared the efficacy of EB (FDA primary selective enrichment broth), UVM (USDA primary selective enrichment broth), MUVM and Frazer broth to recover heat treated cells. The authors observed either no change or a decrease in the number of cell survivors after 8 hours enrichment, where a greater recovery of injured cells occurred in EB, MUVM, UVM

and FB respectively. In addition, supplementation of the enrichment media with catalase enhanced the recovery of these treated cells.

Among the numerous researchers that compared the efficacy of various *Listeria* enrichment procedures, Crawford *et al.* 1989, Bailey *et al.* 1990a, McCarthy *et al.* 1990, Lovett *et al.* 1991, Warburton *et al.* 1992 and Potel and Beuchat 1995 studied the recovery of heat treated *Listeria* cells from foods. This study supports the findings of McCarthy *et al.* (1990) where these authors demonstrated no significant difference between the FDA and USDA detection methods for the recovery of heat treated cells in shrimp. While Westoo and Peterz (1992), Hayes *et al.* (1992) and Warburton *et al.* (1992) did not find any significant difference between these 2 enrichment methods, the latter authors demonstrated that recovery of *Listeria* cells was significantly better when used in combination, compared to using only one method.

This study revealed that the most significant difference in the level of cell recovery was observed over first 12 to 16 hours of enrichment using either reconstituted infant milk formula or TSYEB as the resuscitation medium. While both the smooth and rough forms responded similarly to the selection of resuscitation broths under study, a greater concentration of smooth form cells were detected at each sample time period. This difference in recovery patterns was possibly due to the final rough form cell concentration not being standardised prior to heating (i.e. the smooth and rough final cell concentrations differing by a factor of 10).

The results of Amoako *et al.* (1992) confirmed that TSYEB recovered greater numbers of injured cells compared to the primary *Listeria* selective enrichment broth (FDA). Furthermore this author revealed that these heat treated cells were fully recovered within 6 to 8 hours of enrichment. Bailey *et al.* (1990a) demonstrated however, that only the UVM consistently allowed recovery of heat treated *L. monocytogenes* cells compared to EB (the presence of glucose and the lack of adequate buffering system in EB accounting for this difference). However, the efficacy of reconstituted infant milk formula as a non-selective primary enrichment stage has not been addressed by these or other research groups.

Supplementation of the FDA and USDA recommended enrichment techniques with an initial TSYEB or tyndallised IMF non-selective pre-enrichment stage significantly

improved the detection of heat injured cells in this study. The results revealed that these 2 recommended recovery techniques failed to resuscitate cells that were held at 62.8°C for longer than 5 mins, while heat treated cells pre-enriched in either TSYEB (for 10 hours) or reconstituted IMF (for 24 hours) prior to selective enrichment were recovered after 10 and 20 minutes respectively.

Although there was no significant difference between the FDA and USDA techniques in relation to the levels of cell recovery, the use of reconstituted IMF as an pre-non selective enrichment stage provided superior recovery compared to omitting a non-selective enrichment stage prior to the recommended selective enrichment methods.

The greatest survival of heat injured cells occurred when both surface culture forms were cultivated at 41°C prior to heat treatment. Furthermore, the rough form cultivated at this elevated temperature was detected at longer holding times when IMF was employed as the primary non-selective pre-enrichment stage. This study demonstrated that a secondary enrichment stage did not improve the detection of either rough or smooth cells which had been heat treated without the presence of competing bacteria.

In conclusion, any attempt to recover heat treated *Listeria* cells from contaminated IMF should be performed using a pre non-selective enrichment stage in conjunction with a recommended selective enrichment method.

7.4 Isolation and identification of *Bacillus* to species level from IMF.

A practical identification protocol for the isolation and presumptive identification of *Bacillus* to species level from IMF was achieved in this study. The study initially described a method of reconstituting, cooling and storing IMF (under aseptic conditions) so that the microbiological quality of these products could be subsequently assessed under varying conditions of preparation and storage.

Identification of the genus *Bacillus* to species level involved performing a sequence of defined morphological and/or biochemical tests which could at best be described as arduous, labour, time and consumable intensive. As the genus *Bacillus* is one of 13 bacterial genera which form endospores, this study initially identified certain key

morphological, physiological and/or biochemical tests which distinguished the presumptive *Bacillus* isolates from these other 12 genera. These properties included:, a catalase positive reaction (although there are a few exceptions), the production of endospores under aerobic conditions and failure to grow in culture media (pH 3) which had been incubated at 45°C.

Based on the morphological, physiological and/or genetical information provided by Smith *et al.* (1952); from the contributions of other researchers (Sneath 1962, Wolf and Burke 1968, Gordan *et al.* 1973, Bonde 1975, Boeye and Aerts 1976, Logan and Berkeley 1981, Sneath and Jones 1986, Priest and Alexander 1988, Parry *et al.* 1988, Gordan 1989, Ash 1991 and Berkeley and Ali 1994) and from studies carried out in this laboratory, an identification scheme based on the assignment of *Bacillus* to 1 or 3 morphological groups was developed. Validation studies employing 13 typed *Bacillus* strains (obtained from the NCTC and/or NCIMB) confirmed that this procedure successfully identifies presumptive *Bacillus* isolates to species level.

The procedure initially established that the unknown organism was and aerobic spore forming possibly belonged to the genus *Bacillus*, identification to species level was achieved by employing a series of conventional morphological and biochemical tests. The presumptive *Bacillus* isolate was then assigned to 1 of 3 morphological groupings (based on its spore shape and whether or not this spore caused the sporangium to swell, and other related physiological characteristic properties) using a flow scheme developed during this study. As each *Bacillus* spp. has a unique set of physiological and morphological properties, this flow scheme illustrated a sequence of key morphological and/or biochemical tests which facilitated identification to species level (i.e. the results of these tests facilitating the separation and differentiation of closely related species).

After tentatively identifying the type of *Bacillus* present, confirmation of this identification was achieved by carrying out a number of additional tests described in 1 of 4 tables (each table illustrated a set of properties unique to the individual species) and by performing an API 50 CHB/ API 20 E profile analysis (the results of which were interpreted using the APIlab software package).

This commercially available software package revealed whether the identification was unacceptable (i.e. the organism not belonging to the genus *Bacillus*), acceptable to genus level (i.e. due to the large variation in physiological/morphological properties the organism would not be confidently assigned to a particular species), good or excellent identification to species level. The greater the number of biochemical/fermentation reactions which coincided with the reference reaction profile stored in the software package for that particular *Bacillus* spp., the more confident the identification results profile would be.

Logan and Berkeley (1981), Bryant *et al.* (1985) and the International Committee on Systematic Bacteriology- Subcommittee on the taxonomy of the genus *Bacillus* (1995) evaluated the efficacy of the API 50 CHB and API 20 E and found that these biochemical/fermentation test kits assist in the identification of the *Bacillus* to species level.

If the identification procedure failed to assign the aerobic spore former to one of the 3 morphological groupings on the basis of the morphological and/or physiological tests applied, then further tests were carried to establish whether the unknown isolate belonged to the unassigned (*incertae sedis*) group of *Bacillus*.

In conclusion, the study revealed that there was no fast method for the identification of unknown aerobic spore formers due to the large variation in morphological and physiological properties between members of the genus *Bacillus*, and the very close similarity in these properties between closely related species. However, the identification scheme could be confidently employed to identify presumptive *Bacillus* spp. detected in reconstituted IMF in the subsequent studies.

7.5 Microbiological quality of reconstituted infant milk formulae analysed under conditions of preparation and storage abuse.

During the course of this present study, 100 reconstituted IMF (comprising of 10 different brands and purchased from local retail outlets in Glasgow over a 12 month period) were examined in order to determine the effect of various preparation, cooling and storage conditions on; (a) the type and number of indigenous *Bacillus* spp.

present and (b) the detection of heat treated *Listeria* cells which may have contaminated the powder.

Principle results revealed that all 100 IMF sampled immediately after reconstitution were of satisfactory microbiological quality, as all the samples had a Total Aerobic Mesophilic Counts of $\leq 1.0 \times 10^4$ CFU g^{-1} powder and a *Bacillus cereus* Count of $\leq 1.0 \times 10^3$ CFU g^{-1} powder (ICMSF 1986). The microbiological quality amongst the 100 samples varied over the range $\leq 1.0 \times 10^2$ to $\leq 1.0 \times 10^4$ CFU g^{-1}, with 48% of samples within 0 and $\leq 1.0 \times 10^2$, 38% within $\leq 1.0 \times 10^2$ and $\leq 5.0 \times 10^2$, 5% within $\leq 5.0 \times 10^2$ and $\leq 1.0 \times 10^3$, 8% within $\leq 1.0 \times 10^3$ and $\leq 5.0 \times 10^3$, 1% within $\leq 5.0 \times 10^3$ and $\leq 1.0 \times 10^4$ and 0% $> 1.0 \times 10^4$ CFU g^{-1} powder. The overall mean mean Total Aerobic Mesophilic Counts were 3.4×10^2 (range 0 to 6.1×10^3 CFU g^{-1}) and 3.3×10^2 CFU g^{-1} powder (range 0 to 2.6×10^3 CFU g^{-1}), where the IMF were reconstituted with sterile water at temperatures of 56°C and 90°C respectively.

The microbiological quality of infant milk powder purchased in Scotland would appear to be similar to that of Japan and Italy, where Veda *et al.* (1980) analysed 78 dried commercial baby foods in Japan and discovered that these products contained $> 10^2$ aerobic spore forming bacilli g^{-1} powder, and Finoli and Rondini (1989) analysed 26 infant formulae in Italy and found that the Total Aerobic Counts did not exceed 2.0×10^2 CFU g^{-1} powder.

Becker and Terplan (1992) reported that when 261 samples of infant food (distributed in 17 countries) were examined for *B. cereus*, 54% were contaminated with levels between the range 0.3 to 600 CFU g^{-1}. While this study revealed a mean Total Aerobic Mesophilic Counts of 1.6×10^1 (range, <1 to 4.0×10^2 g^{-1}) and 1.4×10^1 (range, <1 to 4.0×10^2 g^{-1}) for *B. cereus I* and *II* recoverd from IMF which had been reconstituted at a sterile water temperature of 56°C. In 1987, a working party of the Scottish Food Co-ordinating Committee reported that in a preliminary survey of bacterial quality of dried milk in Scotland, counts for 12 types of baby formulae ranged from 0 to 4.5×10^6 CFU ml^{-1} (Scottish Food Co-ordinating Committee,1987). This present study reveals that the microbiological quality of dried infant powder has improved over the last decade as the counts did not exceed 6.1×10^3 CFU g^{-1}.

The variation in IMF preparation (i.e. temperature of sterile water used to reconstitute the IMF and whether or not the samples were pasteurised prior to cooling); cooling method (i.e. tap cooling, table top cooling, immediate refrigeration) and type of plating media (i.e. BCSA, BA, NA) did not effect the type and number of organisms present.

The type of organisms predominately isolated from IMF after reconstitution belonged to the *B. subtilis* subgroup, i.e. *B. licheniformis*, *B. subtilis* and *B. pumilus* being detected in 46%, 30% and 6% of all samples analysed. Other organisms making up the indigenous flora of IMF powder included: *B. cereus I* (10%), *B. cereus II* (7%), *B. megaterium* (6%), *B. coagulans* (6%), *B. sphaericus* (8%), *B. amylioliquefaciens* (1%), *B. firmus* (4%), *B. circulans II* (4%), *B. brevis* (1%), *B. polymyxa* (3%), *B. mycoides* (5%) and Gram positive cocci (3%). However, using the manufacturers recommendations for reconstituting the dried infant powder, *Listeria* spp. (including *L. monocytogenes*) were not isolated prior to subsequent storage using either the modified FDA and/or USDA methods.

Lovell (1981), Stadhouders *et al.* (1982), Kwee *et al.* (1986) and earlier work by Crosseley and Johnson (1949) established that the bacterial flora of powdered milk consists primarily of aerobic spore formers, thermoduric cocci and/or *Corynebacteria*. Veda *et al.* (1980) also showed that the most frequently isolated organisms form dried baby food in Japan were *B. licheniformis* and *B. subtilis*, while other *Bacillus* recovered included *B. cereus*, *B. pumilus*, *B. megaterium*, *B. circulans* and *B. coagulans*.

Irrespective of the temperature and duration of storage, this study revealed that the temperature of the sterile water used to reconstitute IMF, subsequent pasteurisation and/or method of cooling did not significantly influence either the type or number of organisms present. However, in samples where the concentration of cells/spores was reduced by exposure to a high reconstitution temperature (90°C), then subsequent heating in the form of pasteurisation or table top cooling were revealed to augment this reduction in cell number (compared to unpasteurised samples prepared at 56°C, and cooled via tap or refrigeration). Furthermore, storage of samples which had been initially reconstituted at 90°C often resulted in the emergence of *Bacillus* spp. (i.e. *B.*

cereus, *B. mycoides*, *B. sphaericus* and *B. megaterium*) which were not identified in the same samples prepared at the lower temperature of 56°C. It is possible that these *Bacillus* spp. emerged from slow germinating endospores which require higher reconstituting temperatures in order to activate germination.

Stadhouders *et al.* (1980) showed that heating of milk at temperatures from 65 to 95°C for various holding times activates *B. cereus* spores (which go on to germinate). This author revealed that there exists slow germinating spores of *B. cereus* (which for the main part do not germinate within 24 hours in HTST milk at 20°C) that require a more intense heat activation compared to fast germinating spores (which successfully germinate within 24 hours at 20°C after an equivalent HTST process).

By far the greatest parameters influencing the microbiological quality of reconstituted and stored IMF products were the temperature and duration of storage. Storage of samples at temperatures ≥20°C for ≥14 hours resulted in a change in the type, number and concentration (CFU g^{-1}) of indigenous *Bacillus* spp. present, with the appearance of organisms (i.e. *B. lentus* and *B. laterosporus*) which were not detected in dried formulae following reconstitution. Indeed, storage of samples for ≥14 hours at ≥25°C resulted in most of the IMF samples exhibiting a Total Aerobic Mesophilic Count $\geq 10^4$ CFU g^{-1}, which is deemed as unsafe for infant consumption (ICMSF 1986). Becker and Terplan (1992) showed that when reconstituted formulae samples with 100 *B. cereus* cells ml^{-1} were incubated at room temperature (27°C), levels of 10^5 organisms ml^{-1} were reached in 7-9 hours. However, this study revealed that the storage of samples (containing a similar starting concentration of *B. cereus*) under various preparation and cooling conditions for 14 hours at 30°C resulted in a maximum count of only $1.4x10^4$ CFU g^{-1}, with a lower mean count of $5.15x10^3$) CFU g^{-1}.

The results from this study showed that the greater the temperature of incubation and/or the longer the duration of storage (at temperatures ≥20°C), the greater the concentration of *Bacillus* observed (CFU g^{-1}). However, storage of samples ≤10°C did not significantly alter the microbiological quality. In addition, storage of samples under conditions of "temperature abuse" (i.e. ≥20°C) resulted in a change in the indigenous flora present.

This present study revealed that *B. cereus I* and/or *II* out competed members of the *B. subtilis* subgroup (which were the dominant *Bacillus* present in dried IMF prior to storage), often growing to their exclusion. Interactions between *B. licheniformis* and *B. cereus* in artificially coinoculated laboratory based media and in IMF supported these findings where the former organism was inhibited once *B. cereus* had reached a cell population of approximately 10^4 CFU ml^{-1}. Irrespective of the preparation, cooling and/or storage conditions employed, *Listeria* spp. (including *L. monocytogenes*) were not isolated from the reconstituted IMF samples during this study.

This study showed that of the 38 *B. cereus II* strains isolated from IMF, only 10 exhibited diarrhoeal enterotoxin production in BHI broth (supplemented with glucose) under orbital conditions (250 rpm) at 30°C. Moreover, of these 38 strains, 5 and 1 (enterotoxin producers) and 12 and 3 (non-enterotoxin producers) exhibited growth on TSYEA and LSA plates at 8°C and 5°C respectively over a 10 day incubation period. This would suggest that of the *B. cereus II* strains isolated from IMF, 5 are of clinical significance as they were capable of toxin production and growth at both psychrotrophic (near refrigeration) and ambient (room) temperatures.

Other authors have also demonstrated that certain members of the genus *Bacillus* are capable of growth at commercial refrigeration temperatures and the incidents where these species have been recovered from reconstituted infant milk formulae are numerous. Credit *et al.* (1992) revealed that *Bacillus* species constituted 84% of the microflora of pasteurised milk stored at 4.5°C over a 30 day observation period. While Coghill and Juffs (1979) revealed that 23.3% of raw milk samples and 16.7% of pasteurised milks were contaminated with aerobic spore formers (i.e. *B. cereus*, *B. megaterium*, *B. licheniformis*, *B. coagulans* and *B. firmus*).

Granum *et al.* (1993) demonstrated psychrotrophic abilities among 15% of the 85 *B. cereus* strains isolated from dairy products (7% of which were tested positive for both psychrotrophic growth and enterotoxin production). Christiansson *et al.* (1989), Griffiths (1989) and Champagne *et al.* (1994) showed the potential for toxigenic *B. cereus II* strains to grow at 8 and/or 6°C.

During the course of this study, 25 reconstituted IMF samples supplied by the Special Feeding Unit (Royal Hospital for Sick Children, Yorkhill, Glasgow) were analysed for microbiological quality. The results of which showed that 23 had a satisfactory Total Aerobic Mesophilc Count of not more than the recommended $1.0x10^4$ CFU g^{-1}, 1 sample exhibited an unacceptable *B. cereus I* count greater than the recommended $1.0x10^3$ CFU g^{-1} (ICMSF 1986), while the remaining sample was not analysed as it failed the package integrity inspection.

Listeria spp. (including *L. monocytogenes*) were not isolated in these samples using the modified FDA and/or USDA recommended enrichment techniques. This inability to recover sublethally injured *Listeria* cells using these optimised enrichment conditions may have been due to, *Listeria* cells not being present prior to infant formula manufacture, *Listeria* cells unable to survive the spray drying process, the temperature of the water used for reconsituting the powder (56°C or 90°C) being detrimental to the survival of this organism.

Results from the storage of these samples at 4°C and/or 25°C supported the findings of the main study, where incubation at 25°C for ≥14 hours resulted in an increase in the type, number and concentration of organisms present. Again, *B. licheniformis* and *B. subtilis* were the most frequently isolated species recovered from early samples and where *B. cereus* co-habitated the sample with either *B. licheniformis* and/or *B. subtilis*, the former organism inhibited the development of these organisms.

Through the years it has been the practice of many clinical laboratories to simply discard isolates of *Bacillus* spp. (which were often described as "inconsequential aerobic spore forming bacteria") other than *B. anthracis* or *B. cereus*, as contaminants of the skin, hair, etc. (Parry *et al.* 1988, Logan 1988, Koneman *et al.* 1992). Indeed, several authors have made reference to the fact that in clinical laboratories throughout the world, many *Bacillus* isolates are being discarded daily which were in fact of unappreciated relevance to the infections from which they were isolated (Gordan *et al.* 1973, Gilbert *et al.* 1981, Norris *et al.* 1981, Gordan *et al.* 1983).

Of the aerobic spore-formers isolated from the dried IMF samples during this study, *B. subtilis, B. licheniformis, B. pumilus, B. cereus I* and *II, B. mycoides, B. brevis, B.*

megaterium, *B. circulans*, and *B. coagulans* have been implicated in either foodborne related illness and/or opportunist infections (Logan 1988, Parry *et al.* 1988, Koneman *et al.* 1992 and Jackson 1995).

Indeed, baby foods are now considered to be a food class of high risk due to the high susecptibility of the consumer population to enteric pathogens (i.e. under developed immune system), severe response to toxins and increased mortality (ICMSF 1986). Considering the above, the results of this study strongly recommends that reconstituted IMF should not be stored at ≥20°C for more than 14 hours and all infant feeding apparatus (i.e. feeding bottle and teat) shoud be thoroughly cleaned and sterilised before re-use.

In conclusion, dried infant milk formulae commercially available to Scottish retailers is of satisfactory microbiological quality and should not present any health problems to the consumer population if properly reconstituted under hygienic conditions. In addition, *Listeria* spp. should not pose a threat to bottle feeding infants if the guidelines outlined by the manufacturers are followed, as this opportunistic pathogen was not isolated in the 125 IMF samples analysed in this laboratory under various conditions of preparation and storage abuse. As these infant formulae were not reconstiuted using water at an ambient temperature (which may favour the recovery of sublethally injured cells) prior to analysed for the presence of *Listeria* cells, this study recommends that dried infant powder should be reconstituted at temperatures >56°C.

This present study did reveal that the indigenous bacterial flora of inadequately stored IMF samples (consisting of *Bacillus* spp., some of which were previously implicated in either food related illnesses and/or opportunist infections) may proliferate to unacceptable levels, where they may produce the diarrhoeal enterotoxin. This study recommends that all reconstituted IMF should be consumed within 2 hours of the initial preparation (reconstituted IMF to be only stored in a properly maintained refrigerator for not more than 14 hours or at ambient temperature for not more than 4 hours).

7.6 Diarrhoeal enterotoxin studies.

This study demonstrated that diarrhoegenic enterotoxin producing strains of *B. cereus II* exhibit toxin production in both laboratory based media and reconstituted infant milk formulae, where these culture media either contained or were supplemented with the carbohydrate maltodextrin (i.e a product of the enzymatic hydrolysis of starch). Basal media (and/or IMF) containing an alternative carbon source (e.g. lactose) and/or media containing maltodextin that were artificially inoculated with non-enterotoxin producing strains of *Bacillus*, did not support diarrhoeal toxin production. Indeed, this present investigation revealed a direct correlation between enterotoxin production by indigenous *B. cereus II* strains (i.e. detected via the *Bacilllus cereus* Reverse Phase Latex Agglutination test kit) and the presence of maltodextrin in IMF samples.

Garcia-Arrabis and Kramer (1991) showed that cultivating enterotoxigenic *B. cereus II* strains in BHI broth supplemented with either glucose or starch leads to diarrhoeal enterotoxin production.

Becker and Wong (1994) evaluated the efficacy of this *Bacillus cereus* reverse phase latex agglutination (RPLA) test kit (Oxoid product) and the *Bacillus* diarrhoeal enterotoxin visual immunoassay (Tecra product) and discovered that the former method correctly identifies the L2 component of the haemolysin BL (known to cause fluid accumulation in the rabbit ileal loop test), while the latter test system apparently detects 2 non toxic proteins.

Further work by Granum (1993) demonstrated a good correlation between standard techniques to detect enterotoxin production (i.e. Western Immunoblot technique and the Vascular Permeability reaction) and the RPLA enterotoxin identification test system. Buchanan *et al.* (1992) revealed that there is a very good agreement between detection of heat labile enterotoxin using the RPLA test system and toxin production as analysed via a cytotoxic response in human epidermal carcinoma (Hep-2) and Chinese hamster ovary (CHO) cell lines.

This investigation shows that RPLA test kit successfully detects the presence of diarrhoeal enterotoxin in fat free culture extracts (from a variety of reconstituted

infant milk formula) which had initially supported the growth of a toxigenic strain of *B. cereus II*. The fat free culture extract is a prerequisite for these enterotoxin identification techniques, as the fat content interferes with the successful operation of the immunoassay.

This study showed that a fat free IMF extract could be obtain by the use of either of 2 novel separation techniques. Cultivation of toxigenic *B. cereus II* strains in tyndallised IMF supplemented with maltodextrin, followed by either an ultrafiltration (Filtron® Stirred Cell Ultrafiltration System) or a ultracentrifugation procedure (Intercep® Ultracentrifugation system) provided a fat free extract suitable for enterotoxin analysis. While these separation systems were developed by either Filtron® or Intercep®, they were successfully employed for the first time in this laboratory to separate the fat fraction from cultured IMF.

Griffiths (1990) demonstrated that *B. cereus II* and a number of species (*B. circulans*, *B. lentus*, *B. pumilus*, *B. polymyxa*, *B. carotarum*, *B. mycoides* and *B. thuringiensis*) were capable of producing diarrhoeal enterotoxin in milk (and not infant milk formulae) over a temperature range of 6 to 21°C, as detected by the RPLA test system. The author obtained milk ultrafiltrates suitable for subsequent toxin analysis using an ultrafiltration cell (Chemlab Ltd) with a 300,000 molecular weight cut-off membrane. Alternatively, this researcher obtained the ultrafiltrates by centrifugation of samples in an MPS-1 membrane filtration device (Amicon Ltd) at 2,000 g for 30 mins at 6°C. While this study agrees with the findings of Griffiths (1990), however, different ultrafiltration systems (but similar in principle) were employed to separate the fat content from reconstituted IMF (not milk) for subsequent enterotoxin analysis via the RPLA test system.

Furthermore, the growth of a non-enterotoxin producing strain of *B. licheniformis* for 9 hours prior to the addition of a diarrhoegenic *B. cereus II* strain resulted in an increased production of enterotoxin in IMF samples supplemented with maltodextrin (compared to simply growth of a homogenous *B. cereus II* culture in a similar medium). As no previous work has been carried in this area, it is proposed that cultivation of *B. licheniformis* (prior to the addition of *B. cereus II*) alters the composition of the growth medium by possibly breaking down (and making available)

the maltodextrin to lower readily utilisable sugar units, which possibly provided an environment where *B. cereus II* could produce greater levels of enterotoxin. Greater work is required in this area to bring this to a conclusion.

This study aggrees with the work of Garcia-Arribus and Kramer (1991), Granum *et al.* (1993) Baker and Griffiths (1993), as the supplementation of assay media with glucose provided a suitable environment for both growth of *B. cereus II* and subsequent enterotoxin production (i.e. under conditions of orbital cultivation 250 rpm at 30°C).

In conclusion, this study revealed that the normal indigenous *Bacillus* flora of IMF (often considered as inconsequential aerobic spore formers) may contain enterotoxin producing strain of *B. cereus II*, which under conditions of "storage abuse", may successfully proliferate and liberate a diarrhoeal enterotoxin in improperly stored IMF containing maltodextrin. By supplementing IMF with maltodextin in order to enhance the nutritional value of this product, the IMF industry has indirectly provided an environment where the indigenous bacterial population present may produce diarrhoeal enterotoxins (which may result in a bottle feeding infant suffering a food related poisoning).

7.7 Studies on infant feeding bottle/teat cleaning and sterilisation procedures.

The present study showed that normal cleaning procedures alone did not successfully eliminate the bacterial load in contaminated feeding bottles which previously contained reconstituted IMF that was stored under temperature abuse conditions known to occur in the home and/or hospital. Moreover, the greater the degree of cleaning, the larger the reduction in bacterial cell number (CFU ml^{-1}). However, even the most thorough cleaning procedure did not eliminate the microorganisms present.

Application of normal bottle "sterilisation" treatments, such as the Boots® Complete Baby Feedtime Steriliser (i.e. a chemical sterilising treatment), and the Boots® Feedtime Steam Steriliser and/or Boots® Microwave Feeding Bottle Steam Steriliser

(i.e. heat sterilising treatments) successfully eliminated the microoganisms present in contaminated feeding bottles (originally present at levels as high as 10^6 to 10^7 CFU ml^{-1}).

A brief washing or rinsing of the contaminated feeding bottles in 125 ppm Milton solution did not eliminate *B. cereus II*, however immersion of even the most heavily contaminated bottles and/or teats for 30 mins (in 125 ppm Milton solution) resulted in the bacterial load being reduced to a non-detectable level. In 1972, Soderhjelm carried out a survey of bottle and teat hygiene in Sweden where 127 mothers were supplied with a cleaning protocol (and a protocol for sterilisation using the Milton method). Of the 127 mothers who cleaned and chemically sterilised the used feeding bottles only 3 (2.3%) were found to be of poor quality ($>10^2$ CFU ml^{-1}) after a microbiological assessment (satisfactory quality was considered to be <10 CFU ml^{-1}). However, 41 (32.3%) used feeding bottles and teats which were boiled (i.e. boiling time to be determined by the parent and often ranged from less than 2 mins to above 10 mins) were of poor microbiological quality.

This study revealed that when these cleaned (or partly cleaned) and sterilised bottles were filled with BHI broth and stored for 18 hours (temperatures greater than 25°C), a re-emergence of these aerobic spore formers occurred. The level of cleaning prior to sterilisation influenced the concentration of *B. cereus II* observed in these improperly stored feeding bottles.

In conclusion, thorough cleaning (i.e. rinsing, washing and brushing in warm soapy water) of the contaminated or used infant feeding bottles does not guarantee a bacterial free environment. This study revealed that apart from the indigenous flora present in the freshly prepared infant formulae, an additional source of microorganisms in the form of residual biofilm on the feeding bottle from a previous feed may adulterate or contaminate this new infant formula. This investigation strongly recommends that infant feeding bottles should always be cleaned thoroughly, sterilised and never left to sit at ambient temperatures for long periods when refilled with fresh formulae.

7.8 Antagonistic interactions

7.8.1 Antagonistic interaction in solid media.

The results of this study revealed that 40 of the 178 *Bacillus* spp. (i.e. 22.5%) originally isolated from reconstituted IMF exhibited varying degrees of inhibition against the growth of smooth and rough forms of *L. monocytogenes* on TSYEA plates. A variation in the size of the *Listeria* growth inhibition zone was observed, which depended on the combination of indicator (smooth or rough form of *Listeria*) and test organism (*Bacillus* spp.) employed where the antagonistic interactions ranged from a weak (0.5 mm) to a very strong (5.0 mm) zone of clearing.

The initial screening of the large number of *Bacillus* (recovered from IMF samples and stored on slope) for possible *Listeria* antagonists was performed using the direct plating antagonistic method described by Batista (1993), where the non-selective agar medium was seeded with approximately 10^5 *Listeria* cells ml^{-1} prior to the stab inoculation of the test organism (*Bacillus*).

Indeed, of the 40 *Bacillus* exhibiting an antagonistic activity against *Listeria* cells, 37 and 38 of these aerobic spore formers inhibited the growth of smooth and rough forms respectively. Furthermore, only 3 of the resulting 40 (7.5%) failed to inhibit both pleomorphic colony forms of *L. monocytogenes*.

The antagonistic *Bacillus* spp. included: *B. cereus I* (9), *B. cereus II* (16), *B. mycoides* (5), *B. megaterium* (1), *B. subtilis* (5), *B. amyloliquefaciens* (2) and *B. licheniformis* (2). The 13 control *Bacillus* spp. (obtained from the NCTC and/or NCIMB) failed to exhibit any antagonism against either morphological form of *L. monocytogenes*. Further confirmatory studies revealed that the combination of indicator inoculum level (i.e. 10^5 *Listeria* cells ml^{-1}), assay medium (i.e. TSYEA), temperature of interaction (i.e. 30°C) and the method of antagonism (i.e. direct using the stab inoculation technique) initially used during the screening of the 178 *Bacillus* spp. for potential antagonistic strains was satisfactory. These findings support that of Batista (1993), where the author demonstrated that these properties also influenced

the zones of inhibition (mm) produced as a result of an antagonistic interaction between a strain of *B. amyloliquefaciens* and *L. monocytogenes* (the latter organism being inhibited) on solid plating media.

Gonsalez *et al.* (1993) observed that different culture media had little effect on the inhibitory activity of lactobacilli. On the other hand, Holland and Roberts (1966) reported that although megacin C formation was difficult or impossible to demonstrate on a nutrient broth agar, large clear inhibition zones were observed when nutrient agar (Oxoid) was used.

A reaction temperature of 35°C was demonstrated to be unsuitable for the identification of antagonistic test organisms, as certain members of the *B. subtilis* group (i.e. *B. licheniformis* and/or *B. subtilis*) over grew or swarmed the assay plate making it too difficult to screen for possible *Listeria* antagonists. The inhibitory activity exhibited by *Bacillus* was shown to be related to the concentration of *Listeria* cells seeded on the assay plates, as the larger the number of *Listeria* cells present (e.g. 10^7 CFU ml^{-1}), the narrower the zone of inhibition observed surrounding the test organisms. The effect of the concentration of indicator organisms on antagonistic assays has been reported by several researchers. Parente and Hill (1992) showed that the effectiveness of enterocin 1146, a bacteriocin produced by *Enterococcus faecium* DPC146 was reduced by increasing the inoculum of *Listeria*.

Attempts to measure the zones of inhibition on Blood Agar (BA), produced as a result of antagonistic interactions between strongly β-haemolytic *Bacillus* spp. (e.g. *B. cereus* and/or *B. amyloliquefaciens*) and *Listeria*, were obscured by zones of red blood cell clearing resulting from this β-haemolytic activity. Furthermore, antagonistic interactions carried out in BHI agar using the direct stab inoculation technique consistently exhibited smaller zones of inhibition compared to the same test and indicator organisms growing simultaneously on TSYEA plates.

Confirmation to the antagonistic activity demonstrated by the 40 *Bacillus* against both smooth and rough forms of *L. monocytogenes* on solid plating media was achieved by using the deferred antagonistic method. The inhibitory process was detected in the 2 assay media, where the bottom TSYEA layer (which had been seeded with Bacillus)

was overlaid with either YSYEA or BHI agar (containing approximately 10^5 *Listeria* cells ml^{-1}).

While the combination of stab inoculation and assay temperature (30°C) was sufficient to observe zones of *Listeria* inhibition, this deferred antagonistic method did not always provide reproducible results. It was often observed that when the soft agar containing *Listeria* was overlaid on the agar surface in which certain *Bacillus* had previously grown, subsequent incubation resulted in this *Bacillus* spreading (making identification of the zones of inhibition difficult). However, a similar antagonistic interaction profile was observed between these deferred assay plates and those of the direct antagonistic method. Batista (1993) also observed that when TSYEA or BHIA soft agar (containing *Listeria*) was overlaid on top a TSYEA layer which had a 24 hours growth of *B. amyloliquefaciens*, quanitification of the zones of inhibition could be obscured by the *Bacillus* overgrowing the plate.

Demonstration of this inhibition activity was also illustrated in the absence of living *Bacillus* on solid media using the membrane filter method developed by (Batista 1993). Quantification of the zones of inhibition was not determined, as an absence or presence of any antagonistic interaction was only required. This finding suggests that the antagonistic activity exhibited by *Bacillus* was not due to the mere presence of *Bacillus* cells, but rather it was possibly due to either the secretion of some metabolic product from the cells and/or alteration of the surrounding culture medium (e.g. reduction in pH).

7.8.2 Antagonistic interactions in semi-solid plating media.

The antagonistic process exhibited by the short listed 8 *Bacillus* spp. against the smooth and rough forms of *L. monocytogenes* was evident in both TSYEA and BHIA semi-solid media. However, unlike the solid plating media where the antagonistic activity was described in terms of a zone of *Listeria* clearing (mm) surrounding *Bacillus*, inhibition in semi-solid media was described in relation to the volume (mm^3) of *Listeria* inhibited by the test organism (i.e. *Bacillus* was seeded on the surface of the semi-solid agar). While Batista (1993) investigated the effect of the antagonistic

B. amyloliquefaciens against the development of suspended *Listeria* cells in semi-solid agar, the author reported the level of inhibition in terms of height (mm) of medium cleared (whereas a volume term would be more appropriate in this instance). This study demonstrated a variation among the test organisms regarding the volume of *Listeria* cells inhibited, some test strains being more inhibitory than others. Indeed, the volume of inhibition was shown to be related to: the type of test strain employed; the concentration of suspended *Listeria* cells and the temperature of interaction.

A consistently high level of antagonism was exhibited by *B. mycoides* (No. 58), *B. subtilis* (No. 132), and *B. amyloliquefaciens* (No. 170), irrespective of the experimental conditions employed. The study failed to identify a significant difference between the assay media in relation to the volume of inhibition experienced at each *Listeria* inoculum level. In addition, the type of *Listeria* colonial form did not influence the level of antagonism produced by the *Bacillus* spp. in the semi-solid media.

A greater volume of *Listeria* growth inhibition was observed when the interaction was performed at 8°C compared to 30°C. Indeed, certain *Bacillus* test strains (Numbers 35, 58, 132, 133 and 170) demonstrated a very strong antagonism against both pleomorphic forms at 8°C, furthermore a compete clearing of the suspended *Listeria* cells occurred at a *Listeria* inoculum level of 10^4 CFU ml^{-1}. However, even at this psychrotrophic temperature, the level of inhibition depended on the concentration of *Listeria* cells present.

From these results it can be concluded that the antagonistic activity exhibited by *Bacillus* is not exclusive to a particular type or form of culture medium. Storage of both organisms at near refrigeration temperatures did not prevent the antagonistic interaction form taking place, rather it often enhanced the volume of *Listeria* growth inhibition observed.

7.8.3 Antagonistic interactions in liquid media.

The results of this investigation revealed that the growth of both typical and atypical morphological forms of *L. monocytogenes* was inhibited by antagonistic strains of

Bacillus when cultivated simultaneously in reconstituted IMF at 30°C. This finding was observed for interactions carried out using the direct and deferred antagonistic methods.

When *Bacillus* and *Listeria* were cultivated simultaneously (direct antagonistic method), the former organism quickly grew to the exclusion of the smooth and/or rough form. Indeed, apart from confirmation that the *Listeria* inoculum had achieved a concentration in approximately $2.0x10^2$ CFU ml^{-1} in the test media at 30°C, *Listeria* cells were not detected over the 10 day observation period, while the *Bacillus* population gradually decreased in cell number.

When the test organism (*Bacillus*) was cultivated 24 hours prior to the addition of *Listeria* (i.e. deferred antagonistic method 1) the latter organism failed to develop over the 10 day incubation period at 30°C. While cultivating either the smooth or rough forms of *L. monocytogenes* for 24 hours prior to the addition of antagonistic *Bacillus* spp. (via deferred method 2), resulted in a gradual reduction in *Listeria* cell number which coincided with a steady (but slow) increase in *Bacillus*. A similar trend was observed by Batista (1993) where she employed a variety of sterilised milks to examine the interaction between *B. amyloliquefaciens* and *Listeria* in liquid media.

Some reports have suggested that a high initial inoculum level of the starter culture is needed to inhibit the growth of the target organism. Schelinger and Luke (1989) were able to demonstrate the killing of a bacteriocin sensitive strain of *Lactobacillus* sake in MRS broth by *Lactobacillus sake* LB 706 only when the bacteriocin producer reached a cell density of about 10^8 ml^{-1}. This result suggested that a large number of cells is required to obtain bacteriocin concentrations sufficient for effective inhibition of the bacteriocin sensitive organism.

This study failed to demonstrate an antagonistic interaction between *Bacillus* spp. and *L. monocytogenes* at an assay temperature of 4°C. As, both the indicator and test organisms decreased in cell number over the 10 day observation period, either the duration and/or the temperature of incubation should have been increased in order to observe possible antagonistic interactions in liquid media under psychrotrophic conditions. Batista (1994) showed that *Listeria* cells were slightly reduced on interaction with *B. amyloliquefaciens* at 4°C over a 28 day observation period.

However, the author revealed that the level of cell reduction was not commensurate with the level of inhibition observed at 30°C, and postulated that the reason for this difference was due to "a weakness of *Bacillus* at low temperature" (although no growth cycle determination was carried out on *Bacillus* at this temperature).

In conclusion, under conditions where low numbers of heat *treated Listeria* cells may have contaminated an infant milk formula, this osmotically and/or heat *treated* organism may either fail to tolerate the harsh xerophilic conditions of the dried powder, or fail to survive subsequent preparation and storage conditions. This study revealed that if either the smooth or rough form of *L. monocytogenes* survives the severe IMF production, storage and reconstitution conditions, it is possible that certain indigenous *Bacillus* spp. may inhibit its development by exhibiting an undefined inhibitory activity.

7.8.4 Antagonistic interactions between *Bacillus* spp. and other microorganisms.

The results of this investigation demonstrated that the inhibitory activity exhibited by the 40 antagonistic *Bacillus spp.* isolated from infant formulae samples was not exclusive to *L. monocytogenes*, as antagonistic interactions occurred between *Bacillus* and the other members of the genus *Listeria* (as detected by the direct stab inoculation technique on solid plating media).

A variation in the level of inhibition (mm) was evident depending on the combination of test and indicator organism employed. Other Gram negative bacteria were not inhibited by these antagonistic *Bacillus* spp. Siragusa (1992) identified a bacteriocin (hiraecin S) produced by *Enterococcus hirae* which was inhibitory against *L. monocytogenes* and other *Listeria* spp. Inhibition of selected Gram negative and other Gram positive bacteria tested did not occur. Lewus and co-workers (1991) described 10 strains of lactic acid bacteria which exhibited an inhibitory activity against 4 strains of *L. monocytogenes*, 2 strains of *Aeromonas hydrophila* and 2 strains of *Staphylococcus aureus* (all target organisms being of a psychrotrophic nature). Inhibition due to acid. hydrogen peroxide, and lytic bacteriophage were

excluded and the inhibitory activity was confirmed to be of a proteinaceous nature as it demonstrated a sensitivity to proteolytic enzymes (i.e. the authors suggest the inbitory substance was probably a bacteriocin).

Spelhaug and Harlander (1989) described how 3 bacteriocin-producing Lactic acid bacteria (i.e. *Lactococcus lactis subsp.* lactis 11454, *Pediococcus pentosaceous* FBB61 and *P. pentosaceous* FBB63-DG2) inhibited the growth of a variety of foodborne pathogens including: *B. cereus, C. perfringens, S. aureus* and 22 representatives of 5 species of *Listeria* (i.e. *L. monocytogenes. L. ivanovii, L. seeligeri, L. innocua* and *L. welshimeri*).

In addition, Buchanan and Klawitter (1992) identified a strain of *Carnobacterium pisciola* (LK5) which inhibited the of 17 of 21 strains of *Listeria* including: *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri* and *L. grayii*. Its activity was not due to either acid or hydrogen peroxide production, but was related to the production of a heat stable bacteriocin.

7.8.5 Nature of the antagonistic activity.

The results obtained in the investigation about the nature of the antagonistic activity produced by certain strains of *Bacillus* spp isolated from IMF, indicated that it appeared not to be due to production of either acid or hydrogen peroxide. It also seemed not to be due to exhaustion of nutrients or to the production of bacteriophages. The results of this research however, strongly indicate that the antagonistic activity of *B. cereus I* (No. 24), *B. cereus II* (No. 58), *B. mycoides* (No. 59) and *B. amyloliquefaciens* (No. 170) may be caused by the production of substances of proteinaceous nature.

However, as only 4 out of the possible 40 antagonistic *Bacillus* were examined for the nature of the antagonistic activity, it is possible that the inhibitory activity produced by the remaining 36 *Bacillus* spp. may have been due to one or all of the above conditions.

Sirgagua (1992) identified a strain of *Enterococcus hirae* which was found to be inhibitory against *L. monocytogenes* and other *Listeria* spp. The author identified a

bacteriocin, designated as hiraecin S (which was inactivated by pronase and papain), as being the antimicrobial agent involved in this antagonistc interaction. The antimicrobial activity was not due to hydrogen peroxide or acid formation, nor was lysozyme or muramidase activity observed in cell-free bacteriocin preparations.

Lewus *et al.* (1991) described how a bacteriocin/s produced by 10 strains of Lactic acid bacteria inhibited the development of *L. monocytogenes*, *A. hydrophilia* and *S. aureus* under psychrotrophic growth conditions.

Christensen and Hutkins (1992) revealed that the inhibitory action of pediocin JD (produced by *Pediococcus acidolacti* JDI-23) against *L. monocytogenes* is directed at the cytoplasmic membrane and that inhibition of *L. monocytogenes* may be caused by the collapse of one or both of the individual components of the proton motive force.

In a review of the 'bacteriocins of Gram positive bacteria', Jack and his co-workers (1995) described these bactericidal agents as ribosomally synthesised peptides of 30 to less than 60 amino acids, with a narrow to wide antibacterial spectrum against Gram positive bacteria; the antibacterial property is heat stable, and a producer strain exhibits a degree of specific self protection against its own antibacterial peptide. In general, they appear to be translated as inactive prepeptides containing an N-terminal leader sequence and a C-terminal propeptide component. Following post-translational modification (depending on the pH), the molecules may be either released into the environment or remain bound to the cell wall.

The antibacterial action against a sensitive cell of a Gram positive strain is produced principally by destabilisation of the cytoplasmic membrane functions. Indeed, both the lantibiotic (e.g. Nisin A) and non-lanthionine containing bacteriocins (e.g. Pediocin AcH) seem to affect the membrane permeability barrier by forming water-filled membrane channels or pores, probably by a barrel-stave mechanism (Jack *et al.* 1995). These authors further described how various isolates of *L. monocytogenes* were killed as a result of an interaction with other organisms which produced either lentibiotic and/or non-lenthionine containing bacteriocins. Maftah *et al.* (1993) described how mesentericin Y 105 inhibited the transport of both leucine and glutamate by dissipating $\Delta\Psi$ and inducing efflux of pre-accumulated amino acids in sensitive *L. monocytogenes* cells.

In conclusion, from the results obtained there was insufficient evidence to provide a definite answer regarding the nature of the inhibitory substance or substances. A purification scheme involving sequential steps in either adsorption, cation-exchange chromatography, gel chromatography and/or modifications including reversed phase high pressure liquid chromatography should be carried out in order to identify the nature of the inhibitory substance. Therefore, further work should be carried out in order to bring about a clear and definite conclusion, not only for the 4 *Bacillus* spp. tested but for each antagonistic *Bacillus* spp. identified in this study.

Despite this, the present study has resulted in some noteworthy observations. It has demonstrated that certain indigenous *Bacillus* spp. (isolated from IMF) have the ability to inhibit the growth of different morphological forms of *L. monocytogenes*, in addition to the growth of the other members of the genus *Listeria.* The identification of bacteriocin-like products (produced by *Bacillus* isolated from dried infant formulae) which inhibits the growth of potential foodborne pathogens (such as *L. monocytogenes*) offers a further opportunity for both the food industry and the medical profession to continue their fight against unwanted bacterial infections and intoxications. It is evident that the bacteriocin-like products of Gram positive bacteria, especially those with a relatively broad antibacterial spectrum, will continue to be an area of active research.

8. Summary

The principle findings of this research are as follows.

During the course of heating studies, rough cell and colony forms of *L. monocytogenes* emerged which were shown to be significantly different from the smooth form via the image analysis technique. Indeed, rough morphological variants differed by way of producing long cell chains (up to 45 μm in length), in addition the single and paired rods which were consistently seen in the smooth form. This pleomorphic culture form also exhibited different colonial and fermentative characteristics.

The current enrichment and isolation media were efficient at recovering both the smooth and rough morphological forms of *L. monocytogenes*. Of the commercial biochemical/fermentation test systems analysed, both the API *Listeria* and MICRO-ID *Listeria* successfully identified both culture forms to species level, while the API Coryne biochemical gallery identified these culture variants to the genus level only.

The optimum identification protocol for *L. monocytogenes* involved the use of both conventional (i.e. Gram staining, tumbling motility, CAMP test reaction, Henry's oblique illumination technique and testing for catalase activity) and rapid (i.e. API *Listeria* and MICRO-ID *Listeria*) tests.

Factors revealed to significantly improve the heat resistance of *L. monocytogenes* include, cultivation of cells at 40.2, 41 or 42°C in a stationary position (0 rpm) prior to heat treatment, the composition of the cultivation and heating menstruum, the concentration of cells at the point of heat treatment and the temperature and duration of heat inactivation.

The factors shown to influence the recovery of both healthy and sublethally injured cells include; the cultivation temperature and whether or not the cells were shaken, the concentration of cells at time of heating, the temperature and duration of heat treatment, the composition of the cultivation, heating menstruum, diluent, enrichment and enumeration medium (i.e. having a similar media composition at each of these stages) and whether or not the test culture was enriched under gyrotary (110 rpm) or static (0 rpm) conditions. This study revealed that improved recovery of

uninjured/injured cells was not a direct result of these factors working independently, rather, recovery of cells was observed as accumulative effect of these individual factors.

Serotype 4b strains of *L. monocytogenes* (i.e. the serotype implicated as the aetiological agent in a number of food borne outbreaks of listeriosis) was revealed to be more heat tolerant compared to the serotype 1a strain.

The thermal death rates of both the smooth and rough morphological forms did not follow a linear plot, rather both culture forms exhibited a curved thermal death rate. The R-form survivor curve differed from the smooth by exhibiting a tail section, in addition to the initial shoulder section seen in the smooth form. Linearisation of the smooth curved survivor plots was achieved by application of the formula $(\log No - \log N)^a = kt+c$, and a measure of the thermal resistance was obtained by computation of reciprocal of the thermal death rate constant value k (which is analogous to the logarithmic D value, when $c = 0$).

The survivor curve of rough culture variants could not be linearised by application of this formulae. However, this pleomorphic culture form was revealed to be more heat resistant than the smooth by virtue of the fact that it consistently survived longer exposures at sublethal temperatures in a variety of test media.

Pre-enrichment of *L. monocytogenes* for 10-16 hours in either TSYEB or tyndallised infant milk formulae improved the efficacy of the current FDA and USDA selective enrichment techniques, as greater numbers of sublethally injured and healthy cells were recovered. The recommended selective enrichment broths (with or without antibiotic supplements) were revealed to be inferior at recovering heat treated cells compared to the other test media under study.

This study developed a practical identification method for *Bacillus* spp., where presumptive *Bacillus spp.* (isolated from reconstituted infant milk formulae) were successfully identified to species level. This *Bacillus* identification procedure was validated by confirming the identity of 13 typed strains of *Bacillus* which were obtained from either the NCTC and/or the NCIMB culture collections. The microbiological quality of 125 infant milk formulae (purchased from local retail

outlets in Glasgow, Scotland) was analysed under various conditions of reconstitution, cooling and storage.

All these dried infant powders were revealed to be of satisfactory microbiological quality, as they exhibited a Total Aerobic Mesophilic Count and Total *Bacillus cereus* Count within the specifications of the ICMSF. Conditions such as, temperature of the water used to reconstitute these dried powders, the cooling method and subsequent pasteurisation did not significantly influence the type, number and/or concentration (CFU ml^{-1}) of organisms found in infant milk powder. While the temperature (≥20°C) and duration (≥14 hours) of storage of the reconstituted infant powder significantly influenced the number and type of bacteria present. Indeed, the greater the temperature and duration or incubation, the greater the change in the microbiological quality of these baby food products.

The microbiological flora of reconstituted IMF consisted predominantly of *Bacillus* spp., where the dominant flora of the dried powder (prior to reconstitution and storage) consisted of members of the *B. subtilis* group (i.e. *B. licheniformis, B. subtilis* and *B. pumilus*). Other *Bacillus* spp. recovered from the dried powder include, B. *cereus I, B. cereus II, B. megaterium, B. mycoides, B. sphaericus, B. coagulans, B. circulans, B. amyloliquefaciens* and *B. laterosporus*. Subsequent incubation of these reconstituted products at 20 to 25°C resulted in a change in the number and type of *Bacillus* spp., where *B. cereus I* and *II* came to dominate the bacterial flora present (often growing to the exclusion of members of the *B. subtilis* group). Other *Bacillus* spp. originally isolated from the dried powder increased in number on subsequent reconstitution and incubation. A number of these aerobic spore formers have been previously implicated as the causative agent in either clinical infections/intoxications and/or food related illnesses.

Listeria monocytogenes was not recovered from these baby foods using the 2 optimised enrichment techniques, where these products were reconstituted at the bacteriocidal temperature of 56°C (as recommended by the manufacturers).

This study recovered 38 *B. cereus II* isolates, 10 capable of diarrhoeal enterotoxin production via the BCET-RPLA system (3 of these enterotoxin producers growing at near refrigeration temperatures). Infant milk formula containing maltodextin

supported diarrhoeal enterotoxin production, whereas formulae devoid of maltodextin (or containing lactose as the sole carbon source) did not support any toxin production. A fat free culture extract of IMF (a prerequisite for BCET-RPLA analysis) was successfully obtained by way of 2 novel ultrafiltration systems. Thorough cleaning procedures do not eliminate *B. cereus* propagules from contaminated infant feeding bottles. However, cleaning supplemented with a sterilisation eliminated *B. cereus* from contaminated infant feeding bottles.

Antagonistic studies between the 178 *Bacillus* spp. (isolated from reconstituted infant formulae) and *L. monocytogenes* revealed that 22.5% of *Bacillus* exhibited various levels of antagonism against both morphological forms of *Listeria*. Factors shown to influence the level of *Listeria* inhibition include, the concentration (CFU ml^{-1}) of *Listeria*, the type of *Bacillus* spp., the composition of the assay medium, the temperature of interaction, the antagonistic method employed and the method of *Listeria* inoculation.

This antagonistic activity was demonstrated in solid, semi-solid and liquid media. This study further revealed that the antagonistic *Bacillus* exhibited various levels of growth inhibition against other members of the genus *Listeria*. The study was concluded with an investigation into the source of this antagonism (which was revealed to be of a proteinaceous nature).

Listeria spp. (including *L. monocytogenes*) do not pose a significant threat to bottle fed infants in Scotland, once the manufacturers recommendations for preparation and storage of these products are followed. Under conditions of 'storage abuse' the microbiological flora of reconstituted infant milk formulae may pose a significant threat to the health and well-being of bottle fed infants. Finally, a number of *Bacillus* isolates (recovered from infant formulae) may provide a potential source of new anti-microbial agents.

9. References

Aas, N., Gondrosen, B. and Langeland, G. (1992) Norwegian Food Control Authority's report on food associated diseases in 1990. *SNT-Report* 3. Oslo.

Ahamad, N. and Marth E.H. (1989) Behaviour of *Listeria monocytogenes* at 7, 13, 21, and 35°C in tryptose broth acidified with acetate, citrate or lactic acid. *Journal of Food Protection*, 52: 688-695.

Ahmed, A., Moustafa, M.K. and Marth, E.H. (1983) Incidence of *Bacillus cereus* in milk and some milk products. *Journal of Food Protection*, 46:2:126-128.

Ahn, C. and Stiles, M.E. (1990) Plasmid-associated bacteriocin production by a strain of *Carnobacteririum piscola* from meat. *Applied and Environmental Microbiology*, 56: 2503-2510.

Anton, W. (1934) Kritsch-experimenteller beitrag zur biologie des *Bacterium monocytogenes*, mit besonderer berucksichtigung seiner beziehung zur. *Zentralblatt fur Bakteriologie Hygiene OriginalB*. 131: 89-109.

Alterkruse, S., Hyman, F., Klontz, K., Timbo, B. and Tollefson, L. (1994). Foodborne bacterial infections in individuals with the human immunodeficiency virus. *Southern Medical Journal*, 87:2:169-173.

Anderson, P.H.R. and Stone, D.M. (1955). *Staphylococcal* food poisoning associated with spray-dried milk. *Journal Hygiene Cambridge*, 53:4:387-397.

Anderton, A. (1993). Bacterial contamination of enteral feeds and feeding systems. *Clinical Nutrition*, 12(Suppl.1):97-113.

Andrews, W. (1992). Manual of food quality control 4. Rev. 1. *Microbiological analysis. FAO Food and Nutrition Paper* 14/4, Rev. 1:221-338.

Archer, D.L. (1989). Response to *Listeria* deserves a fair trial. *Food Microbiology*, 6:192-193.

Ash, C. Farrow, J.A.E., Wallbanks, S. and Collins, M.D. (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit ribosomal RNA sequences. *Letters in Applied Microbiology* 13: 202-206.

Bahk, J. and Marth, E.H. (1990). *Listeriosis* and *Listeria monocytogenes*. In Foodborne Diseases (ed. Cliver,O.C.) Academic Press Inc pp. 247-257.

Bailey, J.S., Fletcher, D.L. and Cox, N.A. (1989). Recovery and serotype distribution of *L. monocytogenes* from broiler chickens in the South-eastern United States. *Journal of Food Protection*, 52:3:148-150.

Bailey, J.S., Fletcher, D.L. and Cox, N.A. (1990a). Efficacy of enrichment media for recovery of heat-injured *Listeria monocytogenes*. *Journal of Food Protection*, 53:6:473-477.

Bailey, J.S., Fletcher, D.L. and Cox, N.A. (1990b). Effect of enrichment media and sampling protocol on recovery of *Listeria monocytogenes*. *Journal of Food Protection*, 53:6: 505-507.

Baird-Parker, A.C. (1994). Foods and microbiological risks. *Microbiology*, 140:687-695.

Baker, J.M. and Griffiths, M.W. (1992). Predictive modelling of psychrotrophic *Bacillus cereus*. *Journal of Food Protection*, 56:8:684-688.

Bankerroum, N. and Sandine, W.E. (1988) Inhibitory action of nisin against *Listeria monocytogenes*. *Journal of Dairy Science* 71:3237-3245.

Bankerroum, N., Ghouati, Y., Sandine, W.E. and Clarke, A.T. (1993). Methods to demonstrate the bactericidal activity of bacteriocins. *Letters in Applied Microbiology*, 17:78-81.

Bannerman, E., Yersin, M-N. and Bille, J. (1992). Evaluation of the Organon-Teknika Micro-1d *Listeria* system. *Applied and Environmental Microbiology*, 58:6:2011-2015.

Barber, M. (1939) A comparative study of *Listeria* and *Erysipelothrix*. *Journal of Pathology and Bacteriology* 48:11-23

Barza, M. (1985). Listeriosis and milk. *The New England Journal of Medicine*, 312:7:438-440.

Batista, C.V.R. (1993) Studies on the cultural properties of smooth and rough forms of *Listeria monocytogenes* and on antagonistic interactions with *Bacillus amyloliquefaciens*. Phd Thesis, University of Strathclyde, Glasgow.

Beams, R.E. and Girard, K.F. (1958). The effect of pasteurisation on *Listeria monocytogenes*. *Canadian Journal of Microbiology*, 4:55-61.

Beckers, H.J., Soentoro, P.S.S. and Delfgou-van Asch, E.H.M. (1987). The occurrence of *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. *International Journal of Food Microbiology*, 4:249-256.

Becker, H. and Terplan, G. (1992) Importance of *Bacillus cereus* in milk and on dairy equipment, *Journal of Applied Bacteriology*. 23:134-137.

Beecher, D.J. and Wong, A.C.L. (1994). Identification and analysis of the antigens detected by two commercial *Bacillus cereus* diarrheal enterotoxin immunoassay kits. *Applied and Environmental Microbiology*, 60:12:614-4616.

Beliard, E., Abgrall, B. and Bourgeois, C.M. (1989) Selection de souches de bacteries lactiques inhibitrices de *Pseudomonas putida*. Essai d'application a laas conservation de la viande hachee. *Sciences des Aliments*, 9:665-684.

Bergere, J.L. and Cerf, O. (1992). Heat resistance of *Bacillus cereus* spores. In *Bacillus cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation No.* 275/1992. pp. 25-35.

Bergere, J.L. (1992). Spore formation and germination of *Bacillus cereus*: the spore cycle. In *Bacillus Cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation* No. 275/1992, pp. 9-13.

Berkeley, R.C.W. and Goodacre, R. (1992). Identification of some common *Bacillus* thermophiles using the API system and a microcomputer. In. *Economic Microbiology volume* 8. A.H. Rose (ed) p251-256.

Berkeley, R.C.W. and Ali, N. (1994). Classification and identification of endospore forming bacteria. *Journal of Applied Bacteriology Symposium Supplement*, 76:15-85.

Berry, E.D., Hutkins, R.W. and Mandigo, R.W. (1991). The use of bacteriocin-producing *Pediococcus acidilacti* to control postprocessing *Listeria monocytogenes* contamination of frankfurters. *Journal of Food Protection*, 54:9: 681-686.

Beuchat, L.R. (1978) Injury and repair of Gram negative bacteria, with special consideration of involvement of the cytoplasmic membrane. *Advances in Applied Microbiology* 23:219-243.

Beumer, R.R., Johnson, A., Croan, Flynn, P. Whippie, D. Kimbell, A. Lawrie, J and Curiale. (1988) Comparative studies of nucleic acid hybridization assay for *Listeria* in foods. *Journal of the Association of Official Analytical Chemists*, 71: 669-673.

Bille, J. and Doyle, M. (1991) *Listeria* and *Erysipelothrix*. pp.287-295. In. W.J Hausler. Jr., Herrman, K.I., Isenberg, H.D. and Shadowy, H.J. (ed) Manual of clinical microbiology 5th Ed. American Society of Microbiology, Washington, DC.

Bille, J., Catimel, B., Bannerman, E., Jacquet, C., Versin, M-N., Caniaxu, I., Monget, D. and Rocourt, J. (1992). API *Listeria*, a new and promising one-day system to identify *Listeria* isolates. *Applied Environmental Microbiology*, 58:6:1857-1860.

Bloomfield, S.F. and Arthur, M. (1994). Mechanisms of inactivation and resistance of spores to chemical biocides. *Journal of Applied Bacteriology*, Symposium Supplement, 76, 915-1045.

Boerlin, P., Rocourt, J., Grimont, F., Grimont, P.A., Jacquet, C. and Piffaretti, J.C. (1992) *Listeria ivanovii subsp. Indoniensis subsp. nov. International Journal of Systematic Bacteriology*. 42:69-73.

Boeye, A. and Aerts, M. (1976). Numerical taxonomy of *Bacillus* isolates from North Sea sediments. *International Journal of Systematic Bacteriology*, 26:4:427-441.

Bolton, F.J. and Powell, S.J. (1992). Detection of *Listeria* in food and food processing environments. *Food Technology* Spring, 43-48.

Bonde, G.J. (1975) The genus *Bacillus*. *Danish Medical Journal*, 22:41-61.

Boucher, M. and Yonekura, M.L. (1986). Perinatal Listeriosis (Early Onset): Correlation of antenatal manifestations and neonatal outcome. *Obstetrics and Gynecology* 68:593-597.

Bower, C.K., McGuire, J. and Daeschel, M.A. (1995) Suppression of *Listeria monocytogenes* colonization following absorption of Nisin onto silica surfaces. *Applied and Environmental Microbiology*, 61:3:992-997.

Brackett, R.E. (1988). Presence and persistence of *Listeria monocytogenes* in food and water. *Food Technology*, April, 163-168.

Brackett, R.E. and Beuchat, L.R. (1991). Survival of *Listeria monocytogenes* in whole egg and egg yolk powders and in liquid whole eggs. *Food Microbiology*, 8, 331-337.

Bradshaw, J.G., Peeler, J.T., Corwin, J.J., Hunt, I.M., Tierney, J.T., Larkin, E.P. and Twedt, R.M. (1985). Thermal resistance of *Listeria monocytogenes* in milk. *Journal of Food Protection*, 48:9:743-745.

Bradshaw, J.G., Peeler, J.T. and Twedt, R.M. (1991). Thermal resistance of *Listeria* spp. in milk. *Journal of Food Protection*, 54:1:12-14.

Breer, E. and Schofpfer, K. (1988). *Listeria* and food. *The Lancet*, October, 1022.

Brock, T.D., Peacher, B. and Pierson, D. (1963). Survey of the bacteriocins of *Enterococci*. *Journal of Bacteriology*, 86:702-707.

Brosch, R., Catimel, B., Milon, G., Buchrieser, C., Vindel, E. and Rocourt, J. (1993). Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immunocompetent mice and its association with typing characteristics. *Journal of Food Protection*, 56:4:296-301.

Brown, C. (1989). The business of food. Glasgow Herald, April 14.

Brown, K.L. (1994). Spore resistance and ultra heat treatment processes. *Journal of Applied Bacteriology Symposium Supplement*, 76, 675-805.

Bryan, F.L. (1969) Infections due to miscellaneous microorganisms. pp223-297. In. H.Riemann (ed). Food-borne infections and intoxication's. Academic Press, New York.

Bryan, F.L. (1983). Epidemiology of milk-borne diseases. *Journal of Food Protection*, 46:7: 637-649.

Bryant, T.N., Capey, A.G. and Berkeley, R.C.W. (1985) Microcomputer assisted identification of *Bacillus species*. Cabios, 1:23-27.

Bubert, A., Kuhn, M., Goebel, W. and Kohler, S. (1992). Structure and functional properties of the p60 proteins from different *Listeria* spp. *Journal of Bacteriology*, 174(24):8166-8171.

Bubert, A., Schubert, P., Kohler, S., Frank, R. and Goebel, W. (1994). Synthetic peptides derived from the *Listeria monocytogenes* p60 protein as antigens for the generation of polycloncal antibodies specific for secreted cell-free *L. monocytogenes* p60 proteins. *Applied Environmental Microbiology*, 60:9:3120-3127.

Buchanan, R.L. and Golden, M.HJ. (1994). Interaction of citric acid concentration and pH on the kinetics of *Listeria monocytogenes* inactivation. *Journal of Food Protection*, 57:7:567-570.

Buchanan, R.L., Stahl, H.G. and Whiting, R.C. (1989). Effects and interactions of temperature, pH, atmosphere, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. *Journal of Food Protection*, 52:12:844-851.

Buchanan, R.L. and Schultz, F.J. (1992). Evaluation of the Oxoid BLET-RPLA kit for the detection of *Bacillus cereus* diarrhoeal enterotoxin as compared to cell culture cytotoxicity. *Journal of Food Protection*, 55:6:440-443.

Buchanan, R.L., Smith, J.L., Stahl, H.G. and Archer, D.L. (1988). *Listeria*: methods, development, research at the Eastern Regional Research Centre, US Department of Agriculture. *Journal of the Association of Analytical Chemists*, 71:3:651-654.

Buchanan, R.L. (1989). *Listeria* as a foodborne pathogen - a reply to L. Cox. *Food Microbiology*, 6:189-194.

Buchanan, R.L. and Klawitter, L.A. (1992) Characterisation of a lactic acid bacterium. *Carnobacterium piscolacks*, with activity against *Listeria monocytogenes* at refrigeration temperatures. *Journal of Food Protection*. 12:199-217.

Budu-Amoako, E., Toora, S., Ablett, R.F. and Smith, J. (1992). Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured *Listeria monocytogenes* cells. *Applied Environmental Microbiology*, 58:9:3177-3179.

Bueno, M.P. (1994). Infant formula and medical diets. *Journal of the Association of Official Applied Chemists International*, 77:1:155.

Bull, A.T., Goodfellow, M. and Woese, C.R. (1992) Biodiversity as a source of innovation in biotechnology. *Annual Reviews of Microbiology*. 46:219-252.

Bulletin of the World Health Organisation. (1980) Infant feeding-a physiological basis.67(suppl.)

Bunning, V.K., Crawford, R.G., Bradshaw, J.G., Peeler, J.T., Tierney, J.T. and Twedt, R.M. (1986). Thermal resistance of intracellular *Listeria monocytogenes* cells suspended in raw bovine milk. *Applied and Environmental Microbiology*, 52, Dec., 1398-1402.

Bunning,V.K., Donnelly, C.W., Peeler, J.T., Briggs, E.H., Bradshaw, J.G. Crawford, R.G. Beliveau, C.M., Tierney, J.T. (1988) Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. *Applied and Environmental Microbiology*, 54:364-370.

Bunning, V.K., Crawford, RG., Tierney, J.T., and Peeler, J.T. (1992). Thermotolerance of heat-shocked *Listeria monocytogenes* in milk exposed to high-temperature, short-time pasteurisation. *Applied and Environmental Microbiology*, 58:6:2096-2098.

Burn (1935), cited in Batista, C.V.R (1993) Studies on the colonial dissociation and antagonistic interactions between *Listeria monocytogenes* and *Bacillus amyloliquefaciens*. PhD Thesis, University of Strathclyde, Glasgow.

Burn, C.G. (1936) "Clinical and pathological features of an infection caused by a new pathogen of the genus *Listerella*. *American Journal of Pathology*, 12:341-348.

Burnett,I.A., Wardley, B.L. and Magee, J.T. (1989) The milk kitchen, Sheffield Children's Hospital, before and after a review. *Journal of Hospital Infection*. 13:179-185.

Busch, S.V. and Donnelly, C.W. (1992). Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocula*. *Applied and Environmental Microbiology*, 58:1:14-20.

Busta, F.F. (1978) Introduction of injury and repair of microbial cells. *Advances in Applied Microbiology*, 23:364-370.

Calhoun, C.L. and Frazier, W.C. (1966) The effect of available water on thermal resistance of three nonsporeforming species of bacteria. *Journal of Applied Microbiology* 14, 416-420.

Carminati, D., Giraffa, G. and Bossi, M.G. (1989). Bacteriocin-like inhibitors of *Streptococcus lactis* agaisnt *Listeria monocytogenes*. *Journal of Food Protection*, 52:9:614-617.

Carpenter, B. and Cerf, O. (1993). Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology*, 75:499-511.

Cassiday, P.K. and Brackett, R.E. (1989). Methods and media to isolate and enumerate *Listeria monocytogenes:* a review. *Journal of Food Protection*, 52:3:1207-214.

Castle, M.T. and Watkins, S.P. (1984) Cow behaviour and health. p272. In. Modern milk production. (ed. M. E. Castle), Farber and Farber, London.

Champagne, C.P., Laing, R.P., Roy, D., Mafu, A.A. and Griffiths, M.W. (1994). Psychrotrophs in dairy products: their effects and their control. *Critical Reviews in Food Science and Nutrition*, 34:1:1-30.

Christensen, D.P. and Hutkins, R.W. (1992). Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. *Applied and Environmental Microbiology*, 58:10:3312-3315.

Christiansson, A., Satyanarayan Naido,A., Nilson, I., Wadstrom, T. and Pettersson, H.E. (1989). Toxin production by *Bacillus cereus* dairy isolates milk at low temperatures. *Applied and Environmental Microbiology*, 55:10:2595-2600.

Christiansson, A. (1992). The toxicology of *Bacillus cereus*. In *Bacillus cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation*, No. 275/1992. pp. 30-35.

Chung, K.T., Dickson, J.S. and Crouse, J.D. (1989) Effects of nisin on growth of bacteria attached to meat. *Applied and Environmental Microbiology*, 55:1329-1333.

Ciesielski, C.A., Hightower, A.W. and Parsons, S.K. (1988) Listeriosis in the United States 1980-1982. *Archives of Internal Medicine*. 148: 1416-1419.

Coghill, D. and Juffs, H.S. (1979) Incidence of psychrotrophic sporeforming bacteria in pasteurised milk and cream products and effect of temperature on their growth. *Australian Journal of Dairy Technology*. 34:150-153.

Collins, R.N., Treger, M.D., Goldsby, J.B., Boring II, J.R., Coohan, D.B., Barr, R.N. (1968). Interstate outbreak of *Salmonella newbrunswick* infection traced to powdered milk. *Journal of the American Medical Association*, March, 203:10:8-124.

Collins, C.H., Lyne, P.M., and Grange, J.M. (1989). Collins and Lyne's Microbiological Methods. (eds. Collins, C.H., Lyne, P.M., and Grange, J.M), Butterworth & Co (Publishers) Ltd.

Collins-Thompson, D.L., Weiss, K.F., Ridel, G.W. and Charbonneau, S. (1980). Microbiological guidelines and sampling plans for dried infant cereals and powdered infant formula from a Canadian National Microbiological Survey. *Journal of Food Protection*, 43:8:613-616.

Cox, L.J., Kleiss, T., Cordier, J.L., Cordeilana, C., Konkel, P. and Rediazzini, C. (1989). *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology*, 6: 49-61.

Cox L.J. (1989). A perspective on Listeriosis. *Food Technology*, December, 52-59.

Crawford, R.G., Beliveau, C.M., Peeler, J.T., Donnelly, C.W. and Kelly Bunning, V. (1989). Comparative recovery of uninjured and heat-injured *Listeria monocytogenes* cells from bovine milk. *Applied and Environmental Microbiology*, 55: 1490-1494.

Crielly, E.H., Anderton,A., and Logan, N. (1992) Studies of the *Bacillus* flora of milk and milk products. *Journal of Applied Bacteriology*. (77): 256-263.

Crosseley, E.L. and Johnson,W.A. (1942) *Netherlands Milk and Dairy Journal*. 36:231.

Davies, F.L. and Wilkinson, G. (1973). *Bacillus cereus* in milk and dairy products. In The Microbiological Safety of Food. (eds. B.C. Hobbs and J.H.B. Christian). Academic Press, London, pp. 57-67.

Dealler, S.F. and Rotowa, N.A. (1991). Rapid screening of colours from *Listeria* selective agar. *Journal of Hospital Infections*, 17:147-150.

de Barjac, H. (1981) Essai de classification biochemique et serological de 24 souches de *Bacillus* de type B. thurigiensis. *Entomophaga*, 8:223.

del Corral, F. and Buchanan, R.L. (1990). Evaluation of the Api-zym system for identification of *Listeria*. *Food Microbiology*, 7:99-106.

Denyer, S.P., Hanlon, G.W. and Davies, M.C. (1993). Mechanisms of microbial adherence. In Microbial Biofilms: Formation and Control. (eds. S.P. Denyer, S.P. Gorman and M. Sussman). Blackwell Scientific Publications, pp. 13-27.

Department of Health and Social Security (1978). Breast feeding. Committee on Medical Aspects of Food Policy: Panel on Child Nutrition. Office of Population Census and Surveys, London, HMSO., 1-26.

de Zoysa, I. (1991) Why promote breast-feeding in diarrhoeal disease control programmes? Health Policy and Planning. 6:371-379.

Dickerson, R.W. (1986) cited In. Bunning, V.K., Crawford, RG., Tierney, J.T., and Peeler, J.T. (1992). Thermotolerance of heat-shocked *Listeria monocytogenes* in milk exposed to high-temperature, short-time pasteurisation. *Applied and Environmental Microbiology*, 58:6:2096.

Dickson, J.S. (1991). Attachment of Salmonella typhimurium and *Listeria monocytogenes* to beef tissue: effects of inoculum level, growth temperature and bacterial culture age. *Food Microbiology*, 8:143-151.

Donnelly, C.W. and Baignet, G.J. (1986) Method for flow cytometry detection of *Listeria monocytogenes* in milk. *Applied and Environmental Microbiology*, 52:689-695.

Donnelly, C.W. and Briggs, E.H. (1986). Psychrotrophic growth and thermal inactivation of *Listeria monocytogenes* as a function of milk composition. *Journal of Food Protection*, 49:12:994-998.

Donnelly, C.W., Briggs, E.H. and Scott Donnelly, L. (1987). Comparison of heat resistance of *Listeria monocytogenes* in milk as determined by two methods. *Journal of Food Protection*, 50:2:14-17.

Donnelly, C.W. (1988). Historical perspectives on methodology to detect *Listeria monocytogenes*. *Journal of the Association of Official Analytical Chemists*, 71:3:644.

Doyle, M.P., Meske, L.M. and Marth, E.H. (1985) Survival of *Listeria monocytogenes* during the manufacture and ripening of camembert cheese. *Journal of Food Protection*, 50:372-378.

Doyle, M.P., Glass, K.A., Beery, J.T., Garcia, G.A., Dollard, D.J. and Schultz, R.D. (1987). Survival of *Listeria monocytogenes* in milk during high temperature, short-time pasteurisation. *Applied and Environmental Microbiology*, 53:7:1433-1438.

Doyle, M.P. (1988). Effect of environmental and processing conditions on *Listeria monocytogenes*. *Food Technology*, April, 169-171.

Doyle, M.P. and Schoeni, W.H. (1986) Selective enrichment procedure for the isolation of *Listeria monocytogenes* from faecal and biologic specimens. *Applied and Environmental Microbiology.* 51:1127-1129.

Edwards, C.A. Parret, A.M., Balmer,S.E., and Wharton,B.A. (1994) Faecal short chain acids in breast fed and formula fed babies. *Acta Paediatrica* 83:459-462.

El-Dairouty, K.R. (1989). *Staphylococcal* intoxication traced to non-fat dried milk. *Journal of Food Protection*, 52:12:901-902.

Eley, A. (1990). New rapid detection methods for *Salmonella* and *Listeria. Food Safety*, 92:28-31.

El-Kest, S.E. and Marth, E.H. (1992). Transmission electron microscopy of unfrozen and frozen/thawed cells of *Listeria monocytogenes* treated with lipase and lysozyme. *Journal of Food Protection*, 55:4:687-696.

Elliker, P.R. and Frazier, G. (1938) Influence of time and temperature of incubation on heat resistance of *Escherichia coli. Journal of Bacteriology*, 36:83-98.

Esrey. S.A. (1990) Food contamination and diarrhoea. *World Health*.pp.19-20.

Estela, L.A. and Soros, J.A. (1993). Comparison of conventional and reversed phage typing procedures for identification of *Listeria* spp. *Applied Environmental Microbiology*, 59:2:617-619.

Fain, A.R., Line, J.E., Moran, A.B., Michelle Martin, L., Lechowich, R.V., Carocella, J.M. and Brown, W.L. (1991). Lethality of heat to *Listeria monocytogenes* Scott A: D-value and Z-value determinations in ground beef and turkey. *Journal of Food Protection*, 54:10:756-761.

Fairchild, T.M. and Fobgeding, P.M. (1993). A proposed nonpathogenic biological indicator for thermal inactivation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 59:4:1247-1250.

Farber, J.M., Snaders, G.W., Dunfield, S. and Prescott, R. (1989). The effect of various acidulants on the growth of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 9:181-183.

Farber, J.M., Sanders, G.W. and Johnson, M.A. (1989). The survey of various foods for the presence of *Listeria* species. *Journal of Food Protection*, 52::7:456-458.

Farber, J.M. and Fabiotto, F. (1992). The effect of acid shock on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 15:197-201.

Farber, J.M. and Peterkin, P.I. (1991). *Listeria monocytogenes*: a food-borne pathogen. *Microbiological Reviews*, 55:3:476-511.

Farber, J.M., Daley, E., Coates, F., Emmons, D.B. and McKellar, R. (1992). Factors influencing survival of *Listeria monocytogenes* in milk in a high-temperature short-time pasteuriser. *Journal of Food Protection*, 55:12:946-951.

Farber, J.M. (1993). Current research on *Listeria monocytogenes* in foods: an overview. *Journal of Food Protection*, 56:7:640-643.

Farmanian, C., Fremy, J.M. and Ciatsse, M. (1994). Effect of temperature on the vegetative growth of type and field strains of *Bacillus cereus*. *Letters in Applied Microbiology*, 19, 414-418.

Farrag, S.A., El--Gazzar, F.E. and Marth, E.H. (1990). Fate of *Listeria monocytogenes* in sweetened condensed and evaporated milk during storage at 7° or 21°C. *Journal of Food Protection*, 53:9:747-750.

Favret, M.E and Yousten, A.A. (1989) Thuricin, the bacteriocin produced by *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, 53:2:206-216.

Fedio, W.M. and Jackson, H. (1989). Effect of tempering on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 9:157-160.

Fenlon, D.R. and Wilson, J. (1988). The incidence of *Listeria monocytogenes* in raw milk from farm bulk tanks in North-East Scotland. *Journal of Applied Bacteriology*, 66:191-196.

Fenlon, D.R., Stewart, T. and Donachie, W. (1995). The incidence, numbers and types of *Listeria monocytogenes* isolated from farm bulk tank milks. *Letters in Applied Microbiology*, 20:57-60.

Ferguson, R.D. and Shelef, L.A. (1990). Growth of *Listeria monocytogenes* in soy milk. *Food Microbiology*, 7: 49-52.

Fernandez Garayayzabel, J.F., Dominguez Rodiguez, L. Vasquez Boland, J.A., Blanco Cancelo, J.L. and Suarez Fernandez, G. (1986). *Listeria monocytogenes* dans le lait pasteurise. *Canadian Journal of Microbiology*, 32:149-150.

Fernandez Garayayzabel, J.F., Dominguez Rodiguez, L. Vasquez Boland, J.A., Rodriguez Ferri, E.F., Briones Dieste, V. and Blanco Cancelo, J.L. (1987). Survival of *Listeria monocytogenes* in raw milk treated in a pilot plant size pasteuriser. *Journal of Applied Bacteriology*, 63:533-537.

Filer, L.J. (1993). Safe foods for infants - the revaluation of milk infant formula and other infant foods. *American Institute of Nutrition Journal of Nutrition.*, 123: 285-288.

Finoli, C. and Rondinni, G. (1989). Evaluation of infant formula contamination in Italy. *Food Chemistry,* 32:1-8.

Fleet, G.H., Karalis, T., Hawa, A. and Lukondeh, T. (1991). A rapid method for enumerating *Salmonella* in milk powders. *Letters in Applied Microbiology*, 13: 55-259.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Branaum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V. and Reingold, A.L. (1985). Pasteurised milk as a vehicle of infection in an outbreak of listeriosis. *The New England Journal of Medicine*, 312:404-407.

Food and Drug Administration (1967). Bacteriological analytical manual. 2nd Ed. Washington D.C.

Food and Drug Administration (1983). Grade A pasteurised Milk ordanace, 1978 Recommendations, Publications No.229 (Revised 1983). United States Department of Public Health Service, Washington D.C.

Foster, S.J. (1994). The role and regulation of cell wall structural dynamics during differentiation of endospore forming bacteria. *Journal of Applied Bacteriology Symposium Supplement*, 76:255-395.

Fox, G.E., Pechman, K.K. and Woese, C.R. (1977) Comparative cataloguing of 16s ribonucleic acid: molecular approach to procaryotic systematics. *International Journal of Systematic Bacteriology*, 27:44-57.

Franklin, J.G. (1970). Spores in milk: problems associated with UHT processing. *Journal of Applied Biology*, 33:180-191.

Fraser, S. and McKain, B. (1995). Temporary reprieve granted to cheesemaker. Glasgow Herald, Wednesday, 16th August.

Frazer, J.A. and Sperber, E.H. (1988) Rapid detection of *Listeria* species in food and environmental samples by esculin hydrolysis. *Journal of Food Protection*. 51:762-765.

Gaillard, J.L., Berche, P., Frehel, C., Gouin, E. and Cossart, P. (1991). Entry of *Listeria* monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram positive cocci. *Cell*, 65:1127-1141.

Gaillard,J.L., Berche, P., Mounier, J., Ricahrd, S. and Sansonetti, P. (1987). In vivo model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte like cell line Caco-2. *Infection Immunology*, 55:2822-2829.

Garcia-Arribas, M.L. and Kramer, J.M. (1991) The effect of glucose, starch and pH on growth enterotoxin and haemolysin production by strains of *Bacillus cereus* associated with food poisoning and non-gastrointestinal infections. International *Journal of Food Microbiology*, 11:21-34.

Gavalachin, J., Tortorello, M.L., Malek, R., Lunders, M. and Batt, C.A. (1991). Isolation of monoclonal antibodies that react preferentially with *Listeria monocytogenes*. *Food Microbiology*, 8:325-330.

Gaya, P., Medina, M. and Nunez, M. (1991). Effect of the lactoperoxidase system on *Listeria monocytogenes* behaviour in raw milk at refrigeration temperatures. *Applied Environmental Microbiology*, 57:3355-3360.

Geis, A., Singh, J. and Teuber, M. (1983). Potential of lactic streptococci to produce bacteriocin. Applied and Environmental Microbiology. 45:205-211.

Gellin, B.G. and Broome, C.V. (1989). Listeriosis. *Journal of the American Medical Association.* 261:9:1314-1320.

George, S.M., Lund, B.M. and Brocklehurst, T.F. (1988). The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 6: 153-156.

Gericke,B., and Thurn,V. (1994) Identification of infant food as a vehicle in a nosocomial outbreak of *Citrobacter freundii*: epidemiological subtyping by allozyme whole cell protein and antibiotic resistance. *Journal of Applied Bacteriology*, 76:553-558.

Ghosh, A.C. (1978) Prevalence of *Bacillus cereus* in the faeces of healthy adults. Journal of Hygiene. 80:233-236.

Gilbert, R.J. and Parry, J.M. (1977). Serotypes of *Bacillus cereus* from outbreaks of food poisoning and from routine foods. *Journal of Hygiene*, 78: 69-74.

Gilbert, R. (1979). *Bacillus cereus* gastro-enteritis. In Food-Borne Infections and Intoxications. 2nd ed. Academic Press Inc.

Gilbert, R.J., Turnbull, P.C.B , Parry, J.M. and Kramer, J.M. (1981). *Bacillus cereus* and other *Bacillus species*: their part in food poisoning and other clinical infections. In. The Aerobic Endospore Forming Bacteria: Classification and Identification. (eds. Berkeley, R.C.W. and Goodfellow, M.)pp.297-314. Academic Press, London.

Gilbert, R.J. and Kramer, J.M. (1986). *Bacillus cereus* food poisoning. In Progress in Food Safety Symposium. (eds. Cliver, D.O. and Cochrane, B.A.). University of Wisconsin-Madison, pp. 85-93.

Gilbert, P. and Allison, D.G. (1993). Laboratory methods for biofilm production. In Microbial Biofilms: Formation and Control. (eds. Denyer, S.P., Gorman, S.P. and Sussman, M.), Blackwell Scientific Publications, pp. 29-49.

Gill, D.A. (1933) "Circling disease: a meningo-encephalitis of sheep in New Zealand. Notes on a new species of pathogenic organism". *Veterinary Records*, 89:258-270.

Gitter, M.R., Bradley, R. and Blampied, P.H. (1980) *Listeria monocytogenes* infection in bovine mastitis. *Veterinary Records*, 107:390-393.

Glatz, B.A. and Goepfert, J.M. (1976). Defined conditions for synthesis of *Bacillus cereus* enterotoxin by fermenter-growth cultures. *Applied and Environmental Microbiology*, 32:3:400-404.

Goepfert, J.M., Spira, W.M. and Kim, H.U. (1972). *Bacillus cereus*: food poisoning organism - a review. *Journal of Milk Food Technology*, 35:4: 213-227.

Goepfert, J.M., Spira, W.M., Glazz, B.A. and Kim, H.U. (1973). Pathogenicity of *Bacillus* cereus. In *The Microbiological Safety of Food*. (eds. Hobbs, H.C. and Christian, J.H.B.), Academic Press, London, pp. 69-75.

Golden, D.A., Beuchat, L.R. and Brackett, R.E. (1988). Evaluation of selective direct plating method for their suitability to recover uninjured, heat-injured and freeze-injured *Listeria monocytogenes* from foods. *Applied and Environmental Microbiology*, 54:6: 1451-1456.

Golsteyn-Thomas, E.J., King, R.K., Burchak, J. and Gannon, V.P.J. (1991). Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Applied Environmental Microbiology*, 57:9:2576-2580.

Gordan, R.E., Hayes, W.C. and Pang, C.H.-N. (1973) The genus *Bacillus*. United States Department of Agriculture. Agricultural Research Service. Agricultural Handbook. No.427. Washington, DC.

Gordan, R.E. (1989) The genus *Bacillus*. W.M.O'Leary (ed) CDR Press Inc. Florida.

Gould, G.W. (1989) Drying, raised osmotic pressure and low water activity. In *Mechanisms of Action of Food Preservation Procedures* ed. Gould, G.W. pp 97-177. Essex: Elsevier Science Publishers.

Gouet, P., Labadie, J. and Serratore, C. (1978) Development of *Listeria monocytogenes* in monoxenic and polyxenic beef mince. *Zentralblatt fur Bakteriologie Hyg. Orig.B.* 166:87-94

Grant, C.C. (1994). Water intoxiciation secondary to incorrectly mixed infant formula. *New Zealand Medical Journal*, Sept., 359-360.

Granum, P.E., Brynestad, S., Kramer, J.M. (1993a). Analysis of enterotoxin production by *Bacillus cereus* from dairy-products, food poisoning incidents and non-gastrointestinal infections. *International Journal of Food Microbiology*, 17: 269-279.

Granum, P.E., Brynestad, S., O'Sullivan, K. and Nissen, H. (1993b). Enterotoxin from *Bacillus cereus*: production and biochemical characterisation. *Netherlands Milk Dairy Journal*, 47: 63-70.

Granum, P.E. (1994). *Bacillus cereus* and its toxins. *Journal of Applied Bacteriology* Symposium Supplement, 76:615-665.

Gray, M.L., Stafseth, H.J., Thorp. Jr., F., Sholl, L.B. and Riley,W.F. (1948). A new technique for isolating *Listerella* from the bovine brain. *Journal of Bacteriology*, 55:471-476.

Gray, M.L., Stafseth, H.J. and Thorp.Jr., F., (1957) Colonial dissociation of *Listeria monocytogenes. Zent. fur Bakt.I.Orig.Band*, 169:378-391.

Gray, M.L. (1960) "Isolation of *Listeria monocytogenes* from oat silage". *Science*, 132:1767-1768.

Greenwood, M. (1992). Food-borne pathogens - isolation and identification. In Conference Presented by Science South West Limited, pp. 1-12.

Griffiths, M.W., Phillips, J.D., West, I.G., Sweetsur, A.W.M. and Muir, D.D. (1988). The quality of skim-milk powder produced from raw milk stored at 2°C. *Food Microbiology*, 5: 89-96.

Griffiths, M.W. (1990). Toxin production by psychrotrophic *Bacillus* spp. present in milk. *Journal of Food Protection*, 53:9:790-792.

Griffiths, M.W (1994). Toxin production by psychrotrophic *Bacillus* spp. present in milk. Hannah Research Institute, Ayr, KA6, 5HL, Scotland, 1-15.

Griffiths, M.W. and Philips, J.D. (1990). Incidence, source and some properties of psychrotrophic *Bacillus spp.* found in raw and pasteurised milk. *Journal of the Society of Dairy Technology*, 43:3:62-66.

Griffiths, M.W. (1992). *Bacillus cereus* in liquid milk and other milk products. In. Bacillus cereus in Milk and Milk Products. *Bulletin of the International Dairy Federation* No. 275, 36-44.

Gunn, R.A. (1979). Bottle feeding as a risk factor for cholera in infants. *The Lancet*, October, 730-733.

Hancock, I., Bointon, B.M. and McAthey, B. (1993). Rapid detection of *Listeria* spp. by selective impedimetric assay. *Letters in Applied Microbiology*, 16: 311-314.

Hansen, N.H. and Riemann, H. (1963). Factors affecting the heat resistance of non-sporing organisms. *Journal of Applied Bacteriology*, 26:3:314-333.

Hartford, T. and Sneath, P.H.A. (1993). Optical DNA-DNA homology in the genus *Listeria*. *International Journal of Systematic Bacteriology*, 43:1:26-31.

Hauge, S. (1950) *Bacillus cereus* as a cause of food poisoning. *Nordisk Hygienisk Tidskrift*. 31:189-206.

Hayes, P.S., Feeley, J.C., Graves, L.M., Ajello, G.W. and Fleming, D.W. (1986). Isolation of *L. monocytogenes* from raw milk. *Applied Environmental Microbiology*, 51:2:438-440.

Hayes, P.S., Graves, L.M., Ajello, G.W., Swaminathan, B., Weaver, R.E., Wenger, J.D., Schuchat, A., Broome, C.U. and The Listeria Study Group. (1991). Comparison of cold enrichment and US Department of Agriculture methods for isolating *Listeria monocytogenes* from naturally contaminated foods. *Applied and Environmental Microbiology*, 57:8:2109-2113.

Hayes, P.S., Graves, L.M., Swaminathan, S., Ajello, G.W., Malcolm, G.B., Weaver, R.E., Ansom, R.R., Deaver, K., Plikaytis, B.D., Schuchat, A., Wenger, J.D., Pinner, R.W., Broome, C.V. and The *Listeria* Study Group (1992). Comparison of three selective enrichment methods for the isolation of *Listeria monocytogenes* from naturally contaminated foods. *Journal of Food Protection*, 55:12:952-959.

Helke, D.M., Somers, E.B. and Wong, A.C.L. (1993). Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and buna-N in the presence of milk and individual milk components. *Journal of Food Protection*, 56:6:479-484.

Henry, B.S. (1933) Dissociation in the genus *Brucella*. *Journal of Infectious Disease*, 52:374-402.

Henry, N.G., Bayne, H.G. and Garibaldi, J.A. (1969). Heat resistance of *Salmonella* : the uniqueness of *Salmonella senftenberg 775W*. *Applied Microbiology*, 17:1:78-82.

Hevin, B., Morange, M. and Favve, R.M. (1993). Absence of an early detectable increase in heat-shock protein synthesis by *Listeria monocytogenes* within mouse mononuclear phagocytes. *Research in Immunology*, 144: 679-689.

Higginbottom, C. (1940). Bacteriological studies of roller-dried milk powders, roller-dried buttermilk and of roller- and spray-dried whey. Hannah Dairy Research Institute, Kirkhill Ayr, 637,143:308-323.

Hitchins, A.D. and Tran, T. (1990). Initial cell concentration and selective media effects on the isolation of *Listeria monocytogenes* from enrichment cultures of inoculated foods. *Journal of Food Protection*, 53:6:502-504.

Holland, I.B. and Roberts, C.F. (1964). Some properties of a new bacteriocin formed by *Bacillus megaterium*. *Journal of General Microbiology*, 35:271-285.

Holtje, J.V. and Tuomanen, E.I. (1991). The murein hydrolases of *Escherichia coli*; properties, function and impact on the course of infections in vivo. *Journal of General Microbiology*, 137: 441-454.

Hoover, L.J., Daeschel, M.A., Stiles, M.E. and Klaenhammer,T.R. (1989) Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. *Journal of Food Protection*, 52:384-387.

Huang, I-Ping D., Youself, A.E., Marth, E.H. and Matthews, M.E. (1992). Thermal inactivation of *Listeria monocytogenes* in chicken gravy. *Journal of Food Protection*, 55:7:492-496.

Huhtanen, C.N. and Jones, C.O. (1989). A procedure for the direct microscopic count of bacteria in non-fat dry milk. *Journal of Food Protection*, 52:6:404-406.

Hunter, P.R. (1991). Application of hazard analytical critical control point (HACCP) to the handling of expressed breast milk on a neonatal unit. *Journal of Hospital Infection*, 17:139-146.

Hurst, A., Hughes, A. and Collins Thompson, D.L. (1974). The effect of sublethal heating on *Staphylococcus aureus* at different physiological ages. *Canadian Journal of Microbiology*, 20:765-768.

Hurst, A. and Gould, G.W. (1983) The bacterial spore. vol 2. Acadamic Press, London.

In-t Veld, P.H., Soentoro, P.S.S., Delfgou-Van Asch, E.H.M., Notermans, S. (1991). Influence of reconstitution on isolation and enumeration of *Listeria monocytogenes*

from milk powder used for reference samples. *Journal of Food Protection*, 54:2: 124-126.

International Commission on Microbiological Specifications for Foods 1.(1986) Their significance and methods of enumeration. Toronto: University of Toronto Press.

International Committee on Systematic Bacteriology-Subcommittee on the Taxonomiy of the genus *Bacillus*. (1995). International Journal of Systematic Bacteriolgy, 45:193-194.

Jack, R.W., Tagg, J.R., and Ray, B. (1995) Bacteriocins of Gram positive bacteria. *Microbiological reviews*, 59:2:171-200.

Jackson, T.C., Acuff, G.R., Lucia, L.M., Prasai, R.K., Benner, R.A. and Terry, C.T. (1993). Survey of residential refrigerators for the presence of *Listeria monocytogenes*. *Journal of Food Protection*, Vol. 56:10:874-875.

Jackson, S.G., Goodbrand, R.B., Ahmed. R., and Rasatiya.S. (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Letters in Applied Microbiology*, (21):103-105.

Jacquet, C., Catimel, B., Brosch. R., Buchrieser, C., Dehaumont, P., Goulet, V., Lepout, A., Veit, P., Rocourt, J. (1995) Investigations related to the epidemic strain involved in the French Listeriosis outbreak in 1992. *Applied and Environmental Microbiology*, 61:6:2242-2246.

Jay, J.M. (1992). Microbiological food safety. *Critical Reviews in Food Science and Nutrition*, 31:3:177-190.

Jeong, D.K. and Frank, J.F. (1994). Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganisms isolated from heat and dairy processing environments. *Journal of Food Protection*, 57:7: 576-586.

Jenkins, D.E., Schultz, J.E. and Matin, A. (1988) Starvation induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. *Journal of Bacteriology*, 170:1910-3914.

Johansen, G.O., Gram, L. and Meyer, A.S. (1994). The combined inhibitory effect of lysozyme and low pH on growth of *Listeria monocytogenes*. *Journal of Food Protection*. 57:7: 561-566.

Johnson, K.M. (1984). *Bacillus cereus* foodborne illness - an update. *Journal of Food Protection*, 47:2:145-153.

Johnson, K.M., Nelson, C.L., and Busta, F.F.(1988) *Journal of Food Science* 48:286-287.

Johnstone, K. (1994). The trigger of mechanism of spore germination: current concepts. *Journal of Applied Bacteriology Symposium Supplement*, 76:175-245.

Jorgensen, F, P.J. Stephens and S. Knochel (1995) The effect of osmotic shock and subsequent adaptation on the thermotolerance and cell morphology of *Listeria monocytogenes*. *Journal of Applied Bacteriology*, 79, 274-281.

Kamplemacher, E.H and van Noorle Jansen, L.M. (1980) Listeriosis in humans and animals in the Netherlands 1958-1977. *Zentralblatt fur Bakterilogie, Parasitenkunde, Infektion Skrankeiten und Hygiene. Abt. I. Orig.0 A.* 2:211-227.

Kathariou, S., Hacker, J., Hof, H., Then, I., Wagner, W., Kuhn, M. and Goebel, W. (1987). Bacterial cytolysins - extracellular proteins and virulence factors. p.141-150. In R. Rott. and W. Goebel. (ed) *Molecular Basis of Viral and Microbial Pathogenesis*. Springer-Verlag, Berlin.

Kathariou, S., Rocourt, J., Hof, H. and Goebel, W. (1988). Levels of *Listeria monocytogenes* Hemolysin are not directly proportional to virulence in experimental infections of mice. *Infection and Immunity*, 56:2: 534-536.

Kawano, J., Shimizu, A., Takeuchi, S. and Kimura, S. (1986) Isolation and use of experimental phages for typing *Bacillus cereus*. *Science Reports of Faculty of Agriculture Kobe University*, 17:1:163-166.

Kerr, K.G., Birkenhead, D., Seale, K., Major, J. and Hawkey, P.M. (1993). Prevalence of *Listeria* spp. on the hands of food workers. *Journal of Food Protection*, 58:6:525-527.

Kerr, K.G., Birkenhead, D., Seale, K., Major, J. and Hawkey, P.M. (1993). Prevalence of *Listeria* spp. on the hands of food workers. *Journal of Food Protection*, 58:6:525-527.

Kim, H.U. and Goepfert, J.M. (1971) Occurrence of *Bacillus cereus* in selected dry food products. *Journal of Milk Dairy Technology*. 34:12-15.

King, D.A., Bayne, H.G. and Alderton, G. (1979). Nonlogarithmic death rate calculations for *Byssochlamys fulva* and other microorgansms. *Applied and Environmental Microbiology*, March, 37:3:596-600.

Kim, C., Swaminathan, B., Cassaday, P.K., Meyer, L.W. and Holloways, B.P. (1991). Rapid confirmation of *Listeria monocytogenes* isolated from foods by a colony blot assay using a oligoxigenin-labelled synthetic oligonucleotide probe. *Applied Environmental Microbiology*, 57:6:1609-1614.

Klinger, J.D., Johnson, A., Croan, D., Flynn, P., Whippe, D., Kimbell, A. Lawrie, J. and Curiale, M. (1988) Comparative studies of nucleic acid hybridisation assay for *Listeria* in foods. *Journal of the Association of Analytical Chemists*. 71:669-673

Koneman,E.W., Stephan, D.A., Janda,W.M., Schreckenberger, P.C., Winn, Jr., W.C. (ed) (1992) Colour atlas and textbook of diagnostic microbiology 4th ed. J.B. Lippincott Company, Philadelphia.

Knabel, S.J., Walker, J.W., Hartman, P.A. and Mendonca, A.F. (1990). Effects of growth temperature and strictly anaerobic recovery on the survival of *Listeria monocytogenes* during pasteurisation. *Applied and Environmental Microbiology*, 56:2:370-376.

Knipschidt, M.E. (1986) Baby food powder. In. Modern dairy techology. Vol.1. Advances in milk processing. Robinson, R.K. (ed) pp.220-224. Elesevier Applied Science Publishers.

Kotwaliwale, N., Sharma, G.P. and Jain, S.K. (1993). Storage stability of commercially available weaning foods. *Journal of Food Science & Technology*, 30,:5: 331-334.

Kornacki, J.L., Evanson, D.J., Reid, W., Rowe, K. and Flowers, R.W. (1993). Evaluation of the USDA protocol for detection of *Listeria monocytogenes*. *Journal of Food Protection*, 56:5:441-443.

Kramer, J.M., Turnbell, P.C.B., Munshi, G. and Gilbert, R.J. (1982). Identification and characterisation of *Bacillus cereus* and other Bacillus species associated with foods and food poisoning. In. Methods for the isolation and identification of food poisoning organisms. (ed. J.E.L. Corry, D. Roberts and F.A. Skinner), pp.261-286. Society for Applied Bacteriology, Technical Series, No.17. Academic Press. London.

Kramer, J.M. and Gilbert, R.J. (1989) *Bacillus cereus* and other *Bacillus* species. In. Foodborne bacterial pathogens. ed. Doyle, M.P. pp.21-70. New York, and Basel. Marcell Dekker.

Kuhn, M. and Goebel, W. (1989). Identification of an extracellular protein *of Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infection and Immunity*, 57:1:55-61.

Kwee, W.S., Dommett, T.W., Giles,J.E., Roberts, R. and Smith, R.A.D. (1986). Microbiological parameters during powdered milk manufacture - variation between processes and stages. *The Australian Journal of Dairy Technology*, March, 3-8.

Lachica, R.V. (1990). Simplified Henry technique for initial recognition of *Listeria* colonies. *Applied Environmental Microbiology*, 56:4:1164-1165.

Lammerding, A.M., Glass, K.A., Gendron-Fitzpatrick, A. and Doyle, M.P. (1992). Determination of virulence of different strains of *Listeria monocytogenes* and *Listeria innocua* by oral innoculation of pregnant mice. *Applied Environmental Microbiology*, 58:12:3991-4000.

Lappin-Scott, H.M., Jass, J. and Costerton, J.W. (1993). Microbial biofilm formation and characterisation. In Microbial Biofilms: Formation and Control. (eds. Denyer, S.P., Gorman, S.P. and Sussman, M.), Blackwell Scientific Publications, pp. 1-11.

Leasor, S.B., Abbas, C.A. and Furstenberg-Eden, R. (1990) Evaluation of UVM as a growth medium for *Listeria monocytogenes*. p39. In Abstract of the 90th Annual Meeting of the American Society of Microbiology. Washington, DC. Anaheim, California, May 12-19 1990 p.284.

Lechowich, R.V. (1988). Microbiological challenges of refrigerated foods. *Food Technology*, Dec., 84-89.

Leifert, C., Li, H., Chidburve, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. and Harbon, A. (1995). Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27, and *Bacillus pumilus* CL 45. *Journal of Applied Bacteriology*, 78: 97-108.

Lewus, C.B., Kaiser, A. and Montville, T.J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied Environmental Microbiology*, 57:6:1683-1688.

Linton,R.H., Pierson, M.D. and Bishop, J.R. (1990). Increase in heat resistance of *Listeria monocytogenes* Scott A by sublethal heat shock. *Journal of Food Production*. 53:11:924-927.

Loessner, M.J., Bell, R.H., Jay, J.M. and Sinelee, L.A. (1988). Comparison of seven plating media for enumeration of Listeria spp. *Applied and Environmental Microbiology*, 54:12:3003-3007.

Loessner, M.J., Goeppal, S. and Busse, M. (1991). The phagovar variability of *Listeria* strains under the influence of virulent and temperate bacteriophages. *Letters in Applied Microbiology*, 23:192-195.

Logan, N.A. (1988). *Bacillus* species of medical and veterinary importance. *Journal of Medical Microbiology*, 25, 157-165.

Logan, N.A. and Berkeley, R.C.W. (1981) Classification and identification of members of the genus Bacillus using API tests. In The aerobic endospore-forming bacteria: classification and identification. R.C.W. Berkeley and M. Goodfellow (ed) pp 105-140. Academic Press, London.

Logan, N.A. and Berkeley, C.W. (1984). Identification of *Bacillus* strains using the API system. *Journal of General Microbiology*,130:871-1882.

Long, K.Z., Wood, J.W., Gariby, E.V., Weiss, K.M., Mathewson, J.J., de la Cabada, F.J., Dupont, H.L. and Wilson, R.A. (1994). Proportional hazards analysis of diarrhoea due to enterotoxigenic *Escherichia coli* and breast feeding in a cohort of urban Mexican children. *American Journal of Epidemiology*, 139:2: 193-205.

Louie,K.K. (1993) Salmonella serotype tennesse in powdered milk products and infant formula-Canada and the United States, *Journal of the American Medical Association*. 270:4:432.

Lovell, H.R. (1981) In.Dairy Microbiology. Volume 1. The microbiology of milk. Robinson. R.K. (ed) Applied Science Publishers, London.

Lovett, J, (1989) *Listeria monocytogenes*. In. Foodborne bacterial pathogens. (ed M.P. Doyle) New York. Marcell Dekker Inc. p284-306.

Lovett, J., Francis, D.W. and Hunt, J.M. (1987). *Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity. *Journal of Food Protection*, 50:3:188-192.

Lovett, J. and Twedt, R.M. (1988). *Listeria. Food Technology*, April, 188-190.

Lovett, J, and Hitchins, A.D. (1988) *Listeria* isolation Chapter 29, Revised October 13, 1988. Bacteriological Analytical Manual. Association of Official Agricultural Chemists, Arlington.

Lovett, J., Francis, D.W., Peeler, J.T. and Twedt, R.M. (1991) Quantitative comparison of two enrichment methods for isolating *Listeria monocytogenes* from sea foods. *Journal of Food Protection*. 54:7-11.

Lucas, A., Lockton,S., Davies,P.S.W. (1992) Randomised trial of a ready-to-feed compared with powdered formula. *Archives in Disease in Childhood*. 67:935-939.

Luck, H., Mostert, J.F. and Husmann, R.A. (1976). Non-sterility of commercial sterilised milk. *South African Journal of Dairy Technology*, 8:2:103-106.

Lund, A.M., Zottola, E.A. and Pusch, D.J. (1991). Comparison of methods for isolation of *Listeria* from raw milk. *Journal of Food Protection*, 54:8:602-606.

M.A.F.F. (1995). The Infant Formula and Follow-on-Formula Regulations 1995. Statutory Instruments, No. 77 (Food), 1-21.

Mackay, B.M. and Bratchell, W. (1989). The heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 9:89-94.

Mackay, B.M., Pritchet, C., Norris, A. and Mead, G.C. (1990). Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. *Letters in Applied Microbiology*, 10:251-255.

Maftah, A., Renault, D., Vignoles, C., Hechard, Y., Bressollier, P., Ratinaud. M.H., Cenatiempo, Y., and Julian, R. (1993). Membrane permeabilization of *Listeria monocytogenes* and mitochondria by bacteriocin mesentericin Y 105. *Journal of Bacteriology*, 175:3232-3235.

Marandi, A., Afzali, H.M. and Hossaini, A.F. (1993). The reasons for early weaning among mothers in Tehran. *Bulletin* of the WHO, 71:5:561-569.

Marquis, R.E., Sim, J. and Shin, S.Y. (1994). Molecular mechanisms of resistance to heat and oxidative damage. *Journal of Applied Bacteriology Symposium Supplement*, 76, 405-485.

Marrakchi, A.El., Hamama, A. and Othmani, F. El. (1993). Occurrence of *Listeria monocytogenes* in milk and dairy products produced or imported into Morocco. *Journal of Food Protection.*, 56:3:256-259.

Marth, E.H. (1993) Growth and survival of *Listeria monocytogenes, Salmonella* species and *Staphylococcus aureus* in the presence of sodium chloride: A review. Dairy Food Environment and Sanitation. 13:14-18.

Martin, J. (1978) Breast feeding, including a summary report of a survey entitled: Infant feeding 1975: attitudes and practice in England and Wales. Office of Population Censuses and Surveys, London, HMSO.

Martin, S.E. and Katz, S.E. (1993) Rapid determination of *Listeria monocytogenes* in foods using a resuscitation/selection/kit system detection, *Journal of the Association of Analytical Chemists*, 76:632-636.

Marugg, J.D. (1991). Bacteriocins, their role in developing natural products. *Food Biotechnology*, 5:3:302-312.

McCallum, K.L. and Inniss, W.E. (1990) Thermotolerance, cell filamentation, and induced protein synthesis in psychrophilic and psychrotrophic bacteria. *Archives of Microbiology*, 153, 585-590.

McCarthy, S.A., Moates, M.L. and McPhearson, R.M. (1990) Recovery of heat stressed *Listeria monocytogenes* form experimentally and naturally contaminated shrimp. *Journal of Food Protection*, 53:183-195.

McCarthy, S.A. (1991). Pathogenicity of nonstressed, heat-stressed and resuscitated Listeria monocytogenes 1A1 cells. *Applied and Environmental Microbiology*, 57:8: 2389-2391.

McClain, D. and Lee, W.H. (1988) Development of USDA method for isolation *of Listeria monocytogenes* from meat. *Journal of the Association of Analytical Chemists*. 71:660-664.

McClain, D. and Lee, W.H. (1989) FSIS method for the isolation of *Listeria monocytogenes* from processed meat and poultry products. Laboratory Commission No.57. Revised May 24, 1989. United States Department of Agriculture, Food Safety and Inspection, Beltsville, M.D.

McKay, A.M. (1990). Antimicrobial activity of *Enterococcus faecium* against *Listeria spp*. *Letters in Applied Microbiology*, 11, 15-17.

McLaughlin,J. (1990). Listeriosis - declining but may return. British Medical Journal, 303:773-775.

Mettler, A.E. (1989). Pathogens in milk powders - have we learned the lesson? Journal of the *Society of Dairy Technology*, 42:2:48-54.

Meyer, D.H. and Donnelly, C.W. (1992) Effect of incubation temperature on repair of heat injured *Listeria* in milk. *Journal of Food Protection*, 55:579-582.

Miles, C.A. and Mackay, B.H. (1994). A mathematical analysis of microbial inactivation oat linearly rising temperatures: calculation of the temperature rise needed to kill *Listeria* monocytogenes in different foods and methods for dynamic measurements of D and Z values. *Journal of Applied Bacteriology*, 77, 14-20.

Minutes of the Meetings, 4/6 July 1994. Postgraduate Medicine and Pharmaceutical Institute, Prague, Czech Republic (1995). International Committee on systematic bacteriology. Subcommittee on the Taxonomy of the Genus *Bacillus*. *International Journal of Systematic Bacteriology*, 45:1:193-194.

Mirhubibolahi, B. (1993). An evaluation of common methods for the detection of *Listeria monocytogenes* in foods by a food examination laboratory. *Food Microbiology* '93 - IUMS-ICMH Conference.

Mossel, D.A.A. (1989) *Listeria monocytogenes* in foods. Isolation, characterisation and control. *International Journal of Food Microbiology*. 8:183-195.

Mosso, M.A., Arribats, M.L.G., Acuena, J. and De La Rosa, M.C. (1989). Enumeration of *Bacillus* and *Bacillus cereus* spores in food from Spain. *Journal of Food Protection*, 52, 184-188.

Motarjemi, Y., Kaferstein, F., Moy, G. and Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organisation*, 71:1, 79-92.

Mounier, J.A., Ryter, M., Coquis-Roudon, M. and Sansonetti, P.J. (1990) Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte like cell line CaCo2. *Infection and Immunity*, 58:1048-1058.

Muir, D.D., Griffiths, M.W., Phillips, J.D., Sweetsur, A.W.M. and West, I.G. (1989). Effect of the bacterial quality of raw milk on the bacterial quality and some other properties of low-heat dried milk. *Journal of the Society of Dairy Technology*, 39:4:115-118.

Murray, E.G.D., Webb,. R.A., and Swann, M.B.R. (1926) A disease of rabbits characterised by large monocular leucocytosis, caused by hitero undescribed bacillus *Bacterium monocytogenes. Journal of pathology and Bacteriology*, 29: 407-409.

Mussaiger, A.O. (1993) Breast feeding patterns and promotion of infant formula in the Republic of Yemen. *Journal of Tropical Pediatrics*, 39:59-63.

Nielsen, J.W., Dickson, J.S. and Crouse, J.D. (1990) Use of bacteriocin produced by *Pediococcus acidilacti* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology*. 56:2142-2145.

Ninet, B., Bannerman, E. and Bille, J. (1992). Assessment of the accuprobe *Listeria monocytogenes* culture identification reagent kit for rapid colony confirmation and its application in various enrichment broth's. *Applied Environmental Microbiology*, 58:12:4055-4059.

Norman Hansen, J. (1994). Nisin as a model food preservative. *Critical Reviews in Food Science & Nutrition*, 34:1, 69-93.

Norme Fil Internationale 143 (1990). Lait et products laitiers, recherche de Listeria monocytogenes., 1-8.

Norris, J.R., Berkeley, R.C.W., Logan, N.A.and O'Donnell, A.G. (1981) The genus *Bacillus* and *Sporolactobacillus*. In. The Prokaryotes: a handbook on habitats, isolation and

identification of *Bacillus*. Ed M.P. Starr, H. Stolp, H.P. Truper, A. Balows and H.G. Schlegel. Vol.2. pp.1711-1742. Springer Verlag, Berlin.

Norrung, B., Solve, M., Ovesen, M. and Skovgaard, N. (1991). Evaluation of an ELISA test for detection of *Listeria* spp. *Journal of Food Protection*, 54:10:752-755.

Northolt, M.D., Bechers, H.J., Vecht, U., Toepel, L., Soentorro, P.S.S. and Wisselink, H.J. (1988) *Listeria monocytogenes*: heat resistance and behaviour during storage of milk and whey and making of Dutch types of cheese. *Netherland Milk Dairy Journal*, 42:207-219.

Northolt, M.D. (1989). Recovery of *Listeria monocytogenes* from dairy products using the TNCB-TNSA method and the draft IDF method. *Netherlands Milk Dairy Journal*, 43, 299-310.

Notermans, S., Chahrabarty, T., Leimeister-Wuchter, M., Dufrenne, J., Heuvelman, K.J., Maas, H., Jansen, W., Wernars, K., Guinee, P. (1989) Specific gene probe for detection of biotyped and serotyped *Listeria* strains. *Applied and Environmental Microbiololgy*, 55:902-906.

Notermans, S. and Tatini, S. (1993). Characterisation of *Bacillus cereus* in relation to toxin production. *Netherlands Milk Dairy Journal*, 47, 71-77.

Nyfeldt, A,. (1929) " Etiologie de la mononuclease infectieuse". *Comptes rendes des seances de la Societe de Biologie*. 101:590-591.

Nyfeldt. A. (1953) Studien uder die *Listerella* Gruppe. Stetpscopio (Roma): 3:249-348. Cited in Gray, M.L., Stafselth, H.J. and Thorp, F.J. (1957) Colonial dissociation of *Listeria monocytogenes, Zent fur.I.Orig. Band.* 169:378-391.

Nygren, B. (1962) Acta. Pathol. Microbiol. Scand. Supp. 160:1-89.

Odlaug, T.E., Jarzynski, V., Caputo, R.A. and Mascoli, C.C. (1982). Evaluation of an automated system for rapid identification of *Bacillus* biological indicators and other *Bacillus* species. *Journal of Parenteral Science and Technology*, 36: 2: 47-54.

O'Donnell, A.G., Norris, J.R., Berkeley, R.C.W., Claus, D., Kaneko, T. Logan, N.A. and Nozaki, R. (1981) Characterization of *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* by pyrolysis gas-liquid chromatography, deoxyribonucleic acid-deoxyribonucleic acid hybridiazation, biochemical tests and API systems. *International Journal of Systematic Bacteriology*, 30: 448-459.

O'Donovan, K. (1959). The occurrence *of Bacillus cereus* in milk and on dairy equipment. *Journal of Applied Bacteriology*, 22:1, 131-137.

Oladepo, D.K., Candlish, A.A.G., and Stimson, W.H. (1992). Detection of *Listeria monocytogenes* using polyclonal antibodies. *Letters in Applied Microbiology*, 14, 26-29.

Ortel, S. (1989) Listeriocins (monocins). *International Journal of Food Microbiology*, 8:249-250.

Owens, J.J., Fitz, F.M. and McDowell, D.A. (1984) Isoenzyme changes during sporulation of Bacillus mycoides, *Process Biochemistry*. 19:2: 63-64.

Oxoid Manual (1990). *Listeria* species and *Listeriosis*. pp. 129-136.

Palmer, S.R. (1990). Epidemiological methods in the investigation of food poisoning outbreaks. *Letters in Applied Microbiology*, 11, 109-115.

Palumbo, S.A. and Willians, A.C. (1989) Freezing and freeze injury in *Listeria monocytogenes*. p.1. In. Abstracts of the 89th Annual Meeting of the American Society for Microbiology. Washington, DC. New Orleans, L.A., May 14-19. 1989, p.319.

Palumbo, S.A. and Williams, A.C. (1990). Effect of temperature, relative humidity and suspending menstru on the resistance of *Listeria monocytogenes* to drying. *Journal of Food Protection*, 53:5:377-381.

Parente, E. and Hill, C. (1992). Inhibition of *Listeria* in buffer, broth, and milk by enterocin 1146, a bacteriocin produced by *Enterococcus faecium*. *Journal of Food Protection*, 44:7:503-508.

Parish, M.T. and Higgins, D.P. (1989) Survival of *Listeria monocytogenes* in low pH model broth systems, *Journal of Food Protection*. 52:144-147.

Parry, J.M., Turnbull, P.C.B. and Gibson, J.R. (1988). A colour atlas of *Bacillus* species. Wolfe Medical Publications Limited.

Patel, J.R. and Beuchat,L.R. (1995) Evaluation of enrichment broths for the ability to repair heat injured *Listeria monocytogenes*. *Journal of Applied Bacteriology*. 78: 366-372.

Paterson, J.S. (1940) Journal of Pathology and Microbiology, 51:427-436.

Pearson, L.J. and Marth, L.H. (1990). *Listeria monocytogenes* - threat to a safe food supply: a review. *Journal of Dairy Science*, 73: 912-928.

Petran, R.L. and Zottola, E.A. (1989) A study of factors affecting growth and recovery of *Listeria monocytogenes* Scott A. *Journal of Food Protection*, 13:458-460.

Pfeifer, J. and Kessler, H.G. (1994). Effect of relative humidity of hot air on the heat resistance of *Bacillus cereus* spores. *Journal of Applied Bacteriology*, 77:121-128.

Philipps, R. (1994). An improved culture/immunoassay for the detection of *Listeria* spp. in foods and environmental samples. *Microbiology Europe*, 2:5:18-23.

Phillips, J.D. and Griffiths, M.W. (1987). The relation between temperature and growth of bacteria in dairy products. *Food Microbiology*, 4:173-185.

Phillips, J.D. and Griffiths, M.W. (1989). An electrical method for detecting *Listeria* spp. Letters in Applied Microbiology, 9:129-132.

Phillips, J.D. and Griffiths, M.W. (1986). Factors contributing to the seasonal variation of *Bacillus* spp. unpasteurised dairy products. *Journal of Applied Bacteriology*, 61: 275-285.

Pirie, J.H.H. (1927) A disease of wild rodents 'Tiger river disease'. Publication of the South African Institute for Medical Research. 3:163-158.

Pirie, J.H.H. (1940) "*Listeria:* change of name for a genus of bacteria". *Nature*, 145:264.

Pisecky,J.(1992) Water activity of milk powders. *Milchwissenschaft*. 47:1:2-7.

Portnoy, D.A, Chakraborty, T., Goebel, W. and Cossart,P. (1992) Molecular determinants of *Listeria monocytogenes* pathogenisis. *Infection and Immunity*. 60:1263-1267.

Potel, J. (1953) The eliology granulomatosis infantiseptica. Wissenschaftliche Zeitschrift der Martin Luther Universitat Halle-Wittenberg III 341,364.

Potel, J. (1963). Active immunisation against Listeriosis with a virulent *Listeria monocytogenes*. *In Second Symposium on Interic Infections*. (ed. Gray, M.L.), Montana State College, Bozeman.

Potel, J. and Schulze-Lammers, J. (1985). *Listeria monocytogenes* vaccine, production and control. *Zentralbl. Bakleriol. Hygiene*, 259:241-248.

Prentice, G.A. and Weaves, P. (1988). *Listeria monocytogenes* in food - its significance and methods for its detection. *Bulletin of the International Dairy Federation*, 223: 1-17.

Prentice, G.A. (1989). Living with *Listeria*. *Journal of the Society of Dairy Technology*, 42:2: 55-58.

Prentice, G.A. and Neaves, P. (1992). The identification of *Listeria* spp. In *Identification Methods in Applied and Environmental Microbiology*. pp. 283-297.

Priest, F.G., Goodfellow, M. and Todd, C. (1981) The genus *Bacillus:* a numerical analysis,p.91-103. In. R.C.W. Berkeley and M. Goodfellow (ed) The aerobic endospore bacteria, classification and identification. Academic Press. Inc., (London).

Priest, F.G., Goodfellow, M., Shute, L.A. and Berkeley, R.C.W. (1987). *Bacillus amyloliquefaciens sp. nov., nom. rev. International Journal of Systematic Bacteriology*, 37:1: 69-71.

Priest, F.G. and Alexander, B. (1988). A frequency matrix for probabilistic identification of some *Bacilli. Journal of General Microbiology*, 134, 3011-3018.

Pucci, M.J., Vedamuthu, E.R. and Vandenbergh, P.A. (1988) Inhibition of *Listeria monocytogenes* by using bacteriocin 1A-1 produced by *Pediococcus acidilacti* 1.0. *Applied and Environmental Microbiology* 54:2349-2353.

Quintavalla, S. and Campanini, M. (1991). Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in heat emulsion. *Letters in Applied Microbiology*, 12, 184-187.

Rajkowski, K.T. and Mikolajcik, E.M. (1986). Characteristics of selected strains of *Bacillus cereus. Journal of Food Protection*. 50, 199-205.

Ralovich, B.S.(1987) Epidemiology and significance of Listeriosis in the European countries, in Schouberg, A. (ed): Listeriosis: Joint WHO:ROI Consultation on Prevention and Control. Berlin. *Instut. fur Veterinarmedizin des Bundesgesundheitsamtes* p21-55.

Rama Raju, V.V. and Kran Kumar, M. (1989). Heat resistance of aerobic spore forming bacteria in milk. *Indian Journal of Dairy Science*, 42, 71-74.

Rayu, V.V.R. and Kumar,M.K (1989) Heat resistance of aerobic spore-forming bacteria in milk. *Indian Journal of Dairy Science*. 12:71-74.

Rea, C.M., Cogan, T.M. and Tobin, S. (1992). Incidence of pathogenic bacteria in raw milk in Ireland. *Journal of Applied Bacteriology*, 73:331-336.

Read, Jr, R.B. (1982). Infant food problems an analytical aspects of providing safe and wholesome infant foods. Association of Analytical Chemists, 65:6:1505-1509.

Rekhif, N., Atrih, A.and Lefebvre, G. (1994). Selection and properties of spontaneous mutants of *Listeria monocytogenes* ACTC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. *Current Microbiology*, 28: 237-241.

Retallack, S.J., Simmer, K., Makrides, M. and Gibson, R.A. (1994). Infant weaning practices in Adelaide: the results of a shopping complex survey. *Australia Paediatric Journal*, 30, 28-32.

Robinson, B.J. and Cunningham, C.P. (1991). Accuracy of MICRO-ID *Listeria* for identification of members of the genus *Listeria. Journal of Food Protection*, 54:10: 798-800.

Rocourt, J., Wehmeyer, U., and Stackebrant, E. (1987) Transfer of *Listeria denitrificans* to a new genus *Jonesia.gen.nov.*, as *Jonesia denitrificans.comb.nov. International Journal of Systematic Bacteriology*. 37:266-270.

Rodriguez, L.D, Fernandez, D.S., Fernandez-Garayzabel, J.F., and Rodriguez Ferri, J. (1984) New metrology for the isolation of *Listeria microorganisms* from heavily contaminated environments. *Applied and Environmental Microbiology*, 47:1188-1190.

Rolston, D.D.K., Raghopathy, P. and Cutting, W.A.M. (1993). Effect of a maltodetrin-electrolyte, a maltodextrin-nutrient-electrolyte and a standard electrolyte fluxes in the secreting rat intestine. *Journal of Diarrhoel Diseases Research*, 11:4:217-221.

Rosenau,M.J. (1909) Milk and its relationship to public health. Hygienic Laboratory Bulletin No.56, US Government Printing Office, Washington,D.C.

Rosenow, E.M. and Marth, E.H. (1987). Growth of *Listeria monocytogenes* in skim, whole and chocolate milk and in whipping cream during incubation at 4, 4, 13, 21 and 35°C. *Journal of Food Protection*, 50:6:452-459.

Rowe, B. (1987). *Salmonella* eating infections associated with consumption of infant dried milk. The *Lancet*, October, 900-903.

Ryser, E.T., Marth, E.H. and Doyle, M.P. (1985). Survival of *Listeria monocytogenes* during manufacture and storage of cottage cheese. *Journal of Food Protection*, 48:9:746-750.

Ryser, E.T. and Richard J.A. (1992). Detection of bacteriocin activity in bacteria using hydrophobic grid membrane filters. *Letters in Applied Microbiology*, 14:104-107.

Ryser, E.T. and Marth, E.H. (1987) Fate of *Listeria monocytogenes* during the manufacture and ripening of camembert cheese. *Journal of Food Protection*. 50:372-378.

Sanz, J., Encianas, J.P., Garcia-Lopez, M.Z., Garcia-Armesto, M.R. and Otero, A. (1993). Evaluation of different systems for the identification of Bacillus strains isolated from Spanish fermented sausages. *Food Microbiology* 93 - ICMSF Conference.

Sazawal, S. Bhan, M.K. and Bhandari, N.(1992) Type of milk feeding during acute diarrhoea and the risk of persistent diarrhoea, a case control study. *Acta Paeditr. Suppl.* 381:93-92.

Schafheitle, J.M. and Light, N.D. (1989). Technical note: sous-vide preparation and chilled storage of chicken Ballotine. 24:199-205.

Schillinger, U., Kaya, M. and Lucke, F.-K. (1991). Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *Journal of Applied Bacteriology*, 70, 473-478.

Schlech III, W.F. (1983). Epidemic listeriosis - endemic for transmission by food. The *New England Journal of Medicine*, 308:4: 203-206.

Schlech III, W.F. (1988). Virulence characteristics of *Listeria monocytogenes*. *Food Technology*, April, 176-179.

Schlech III, W.F. (1991). Listeriosis: epidemiology, virulence and the significance of contaminated foodstuffs. *Journal of Hospital Infection*, 19: 211-224.

Schmitt, N., Bowman, E.J. and Willoughby, B.A. (1976). Food poisoning outbreak attributed to *Bacillus cereus*. *Canadian Journal of Public Health*, 67:418-422.

Schoeni, J.L., Brunner, K. and Doyle, M.P. (1991) Rates of thermal inactivation of *Listeria monocytogenes* in beef and fermented sausage. *Journal of Food Protection*, 54:334-337.

Scottish Food Co-ordinating Committee (1990) Report of a working party on food surveillance. A survey of the bacteriological and chemical qualities of dried milk and related products in Scotland in 1987.

Seeliger, H.P.R. (1961) Listeriosis. New York, Karger.

Seeliger, H.P.R. and Jones, D. (1986) In. "Bergey's Manual of Systematic Bacteriology" 2:1235-1245, Sneath, P.H.A., Maine, N.S., Sharpe, M.E. and Holt. J.G.(ed) Williams and Wilkins, Publications. Baltimore.

Seki, T., Oshima, T. and Oshima, Y. (1975). Taxonomic study of *Bacillus* by deoxyribonucleic acid-deoxyribonucleic acid hybridisation and interspecific transformation. *International Journal of Systematic Bacteriology*, 25:3:258-270.

Sherman, V.B.D, McGowan, V., and Sneath, P.H.A. (1980). Approved lists of bacterial names. *International Journal of Systematic Bacteriology*. 30:225-420.

Shinagawa, K., Konuma, H., Tokumaru, M., Takemasa, N., Hashigiwa, M., Shigehlsa, T. and Lopes, C.A.M. (1988). Enumeration of aerobic spore-formers and *Bacillus cereus* in meat produced additives. *Journal of Food Protection*, 51:8:648-650.

Singh, R.S., Singh, S., Batish, V.K. and Ranganthan, B. (1980). Bacteriological quality of infant milk foods. *Journal of Food Protection*, 43:340-342.

Siragusa, G.R. (1992). Production of bacteriocin inhibitory to *Listeria* species by *Enterococcus hivae*. *Applied and Environmental Microbiology*, 88:1:3508-3513.

Smith, N.R., Gordan, R.E. and Clarke, F.E. (1946) Aerobic mesophilic sporeforming bacteria. Miscellaneous publication No.559. Washington. DC. USDA.

Smith, N.R. Gordan, R.E. and Clarke, F.E. (1952) Aerobic sporeforming bacteria. Monograph No.16. Washington, DC. USDA.

Smith. J.L. and Palumbo, S.A. (1982) Microbial injury reviewed for the sanitarian. *Dairy Food Sanitation*. 2:57-63.

Smith, J.L. and Archer, D.L. (1988) Heat induced injury in *Listeria monocytogenes*, *Journal of Industrial Microbiology*. 3:105-110.

Sneath, H.P.A. (1986) Endospore-forming Gram positive rods and cocci. p.1104-1139. In. Sneath. P.H.A., Mair, N.D., Sharpe, M.E. and Holt, J.G. (ed) section 13, volume 2. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Balimore, London. Los Angeles and Sydney.

Soderhjelm, L. (1972). Infant feeding hygiene in Sweden. *Acta Paediat Scandinavia*, 61, 565-570.

Sorquist, S. (1993). Heat resistance of *Listeria monocytogenes* by two recovery media used with and without cold preincubation. *Journal of Applied Bacteriology*, 74, 428-432.

Sorquist, S. (1994). Heat resistance of different sections of *Listeria monocytogenes*. Journal of Applied Bacteriology, 76: 383-388.

Sorrells, K.M., Enigl, D.C. and Hatfield, J.R. (1989). Effects of pH acidulant, time and temperature on the growth and survival of *Listeria monocytogenes*. *Journal of Food Protection*, 52:8: 571-573.

Spelhaug, S.R. and Harlander, S.K. (1989). Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceous*. *Journal of Food Protection*, 52:12: 856-862.

Spira, W.M. and Silverman, G.L. (1979). The effects of glucose, pH and dissolved-oxygen tension on *Bacillus cereus* growth and permeability factor production in batch culture. *Applied and Environmental Microbiology*, 37:1:109-116.

Stadhouders, J., Hup,g. and Langeveld, C.P.M. (1980) Some observations on the germination, heat resistance and outgrowth of fast germinating and slow germinating spores of *Bacillus cereus* in pasteurised milk. *Netherlands Milk and Dairy Journal*. 34:215-228.

Stadhouders, J. and Hup.G. (1980) Zuivelzicht. 72:178-180. Cited In. Bergere, J.L. (1992). Spore formation and germination of *Bacillus cereus*: the spore cycle. In Bacillus

Cereus in Milk and Milk Products. *Bulletin of the International Dairy Federation* No. 275/1992, pp. 9-13.

Stadhouders, J., Hup, G. and Hassing, F. (1982) *Netherlands Milk and Dairy Journal*. 36:231.

Stadhoulders, J. (1992). Taxonomy of *Bacillus cereus*. In *Bacillus cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation*. No. 275., pp.2-8.

Stadhoulders, J. (1992). The enumeration of spores and vegetative cells of *Bacillus cereus*. In *Bacillus cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation*, No. 275, pp. 15-18.

Stahl, S. (1989) A new bacteriocinogenic activity: megacin B11 encoded by plasmid pSE 203 in strains of *Bacillus megaterium*. *Archives of Microbiology*. 151:2:159-165.

Stanway, P. and Stanway, A. (1983). Best for you. In Breast is Best. (ed. Jolly, H.), Pan Books Limited, Caraye Place, London

Stanway, P. and Stanway, A. (1983). Breast milk - the perfect food. In Breast is Best. (ed. Jolly, H.), Pan Books Limited, Cavaye Place, London, pp. 19-36.

Statutory Instruments, No.77 (1995) Food, The infant formula and follow-on-formula regulations.

Stephan, R., Schraft, H. and Unterman, F. (1994). Characterisation of *Bacillus licheniformis* with the RAPD technique (randomly amplified polymorphic DNA). *Letters in Applied Microbiology*, 18, 260-263.

Stuart, S.E. and Welshimer, H.J. (1974) *International Journal of Systematic Bacteriology*, 24:177-185.

Summer, S.S., Sandros, T.M., Harmon, M.C., Scott, V.N. and Bernard, D.T. (1991) Heat resistance of *Salmonella typhimurium* and *Listeria monocytogenes* in sucrose solutions of various water activities. *Journal of Food Science* 56: 1741-1743.

Surjono, D., Ismadi, S.D , Suwardkji, , and Rohde, J.E. (1980). Bacterial contamination and dilution of milk in infant feeding bottles. *Journal of Tropical Paediatrics*, 58-61.

Svabic-Vlahovic, M. Pantic, D. Pavicic, M. and Bryner, J.H. (1988) Transmission of *Listeria monocytogenes* from mother's milk to her baby and to puppies. *Lancet*. 2:1201.

Thampburan, N. and Surrendran, P.K. (1993). A medium for enumeration of bacteria in foods containing swarming *Bacillus spp*. *Letters in Applied Microbiology* 16: 227-229.

Thomas, L.V., Wimpenny, J.W.T. and Davies, J.G. (1993). Effect of three preservatives on the growth of Bacillus cereus vero cytotoxigenic *Escherichia coli* and *Staphylococcus aureus* on plates with gradients of pH and sodium chloride concentration. *International Journal of Food Microbiology*, 1(17): 289-301.

Thompson, S.S., Harmon, L.G. and Stine, C.M. (1978). Survival of selected organisms during spray drying of skim milk and storage of nonfat dry milk. *Journal of Food Protection*, 41, No. 1, 16-19.

Tomlins, R.I. and Ordal, Z.J (1976) Thermal injury and inactivation in vegetative bacteria. In (eds Skinner, F.A. and Hugo, W.B.) Inhibition and inactivation of vegetative microbes. *Society of Applied Microbiology*, Symposium No.5: 153-184.

Thurn, V. and Gericke, B. (1994). Identification of infant food as a vehicle in a nosocomial outbreak of *Citrobacter freundii*: epidemiological subtyping by allozyme, whole-cell protein and antibiotic resistance. *Journal of Applied Bacteriology*, 76: 553-558.

Tilney, L.G. and Portnoy, D.A. (1989). Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes. Journal of Cell Biology,* 109: 1597-1608.

Tiwari, N.P. and Aldenrath, S.G. (1990). Isolation of *Listeria monocytogenes* from food products on four selective plating media. *Journal of Food Protection*, 53:5:382-385.

Travers, R.S., Martin, W., Phyllis, A., Reicheld-Erfer, C.F. (1987). Selective process for efficient isolation of soil *Bacillus* spp. *Applied Environmental Microbiology*, 53:6: 1263-1266.

Tuncan, E.U. and Martin, S.E. (1989) Combined effect of sucrose and heat treatment temperature on the thermal resistance of *Staphyolococcus aureus* MF-31. *Journal of Food Science*, 54: 936-939.

Turnbull, P.C.B. (1976). Studies on the production of enterotoxins by *Bacillus cereus*. *Journal of Clinical Pathology*, 29:941-948.

Twedt, R.M., Hitchens, A.D. and Prentice, G.A. (1994). Determination of the presence of *Listeria monocytogenes* in milk and dairy products: IDF collaborative study. *Journal of Association of Analytical Chemists International*, 77,:2:395-402.

United States Food and Drug Adminsitration (1983). Grade A pasteurised milk ordinance, 1978 Recommendations, Publications No.229 (Revised 1983). *United States Department Public Health Service*. Washington. D.C.

United States Food and Drug Administration (1967) Bacteriological analytical manual. 2nd Ed. Washington. D.C.

Url, B., Heitzer, A. and Brandl, E. (1993). Determination of *Listeria* in dairy and environmental samples. Comparison of a culture method and a colorimetric nucleic acid hybridisation assay. *Journal of Food Protection*, Vol. 56:7:581-584.

Van de Velde, C., Bounie, D., Cuq, J.L. and Cheftel, J.C. (1984). Destruction of microorganisms and toxins by extrusion cooking. In Thermal Processing and Quality of Foods. (P. Linko, ed.), Elsevier Applied Science Publishers Ltd. Essex

Van Heddeghem, A. and Vlaemynck, G. (1992). Sources of contamination of milk with *Bacillus cereus* on the farm and in the factory. In *Bacillus cereus* in Milk and Milk Products. *Bulletin of the International Dairy Federation* No. 275, 19-22.

Van Netten, P., Van Gaael, B. and Mossel, D.A.A. (1991). Selection, differentiation and counting of haemolytic *Listeria* spp. on palcam medium. *Letters in Applied Microbiology*, 12:20-22.

Van Netten, P., Van de Moosdijk, Van Hoensel, Mossel, D.A. and Peralis,I. (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxin, *Journal of Applied Bacteriology*. 69:73-79.

Van Netten, P. and Kramer, J. (1990). Media for the detection and enumeration of *Bacillus cereus* in foods: a review. *International Journal of Food Microbiology*, 17:85-99.

Vickers, V.T. (1986). Control of airborne contamination in dairy processing plants. *New Zealand Journal of Dairy Science and Technology*, 21:89-98.

Vlaemynck, G. and Van Heddegham, A. (1992). Factors affecting growth of *Bacillus cereus*. In *Bacillus cereus* in milk and milk products. *Bulletin of the International Dairy Federation*, 275, pp. 26-30.

Von Wiese, W. (1969). A contribution to hygiene of the production and preparing of infant formulas and baby foods in infant hygiene. *International Symposium*, 205-210.

Von Wiese, W. (1992). A comparison of microbiological standards: reference values and proposals for dried infant and baby foods.

Walker, S.J., Archer, P. and Banks, J.G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperature. *Journal of Applied Bacteriology*, 68:157-162.

Warburton, D.W., Farber, J.M., Armstrong, A., Caldeira, R., Tiwari, N.P., Babiuk, T., Lacasse, P. and Read, S. (1991). A Canadian comparative study of modified versions

of the "FDA" and "USDA" methods for the detection of *Listeria monocytogenes*. *Journal of Food Protection*, 54:9: 669-676.

Warburton, D.W., Farber, J.M., Powell, C., Tiwari, N.P., Read, S., Plante, R., Babiuk, T., Laffey, P., Kauri, T., Mayers, P., Champagne, M-J., Hunt, T., Lacasse, P., Viet, K., Smando, R. and Coates, F. (1992). Comparison of methods for optimum detection of stressed and low levels of *Listeria monocytogenes*. *Food Microbiology*, 9, 127-145.

Webb, R.A. and Barber, M. (1937) *Listerella* in human meningitis. *Journal of Pathology and Bacteriology*. 45:523-539.

Welker, N.E. and Campbell, L. (1967). Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *Journal of Bacteriology*, 94:4:1124-1130.

Wernars, K., Heuvelman, C.J., Chakraborty, T. and Notermans, S.H.W. (1991) Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. *Journal of Applied Bacteriology*. 70:121-126.

Westhoff, D.C. and Dougherty, S.L. (1981). Characterisation of *Bacillus* species isoalted from spoiled ultrahigh temperature processed milk. *Journal of Dairy Science*, 64: 527-580.

Westoo, A. and Peterz, M. (1992). Evaluation of methods for detection of *Listeria monocytogenes* in foods: NMKL collaborative study. *Journal of the Association of Analytical Chemists International*, 75:1:46-52.

WHO Informal Working Group (1988). Foodborne Listeriosis. WHO/EHE/FOS/88.5, 1-16.

WHO/Hst/92.1. (1992) Globel health situation and projections-estimated/unpublished document. cited In Motarjemi, Y., Kaferstein, F., Moy, G. and Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. Bulletin of the World Health Organisation, 71:1, 79-92.

White, H.P. (1953) The heat resistance of *Streptococcus faecalis*. *Journal of General Microbiology*. 8:27-37.

Wilkinson, G. and Davies, F.L. (1973) *Journal of Applied Bacteriology*. 36:485-496.

Willis, A.T., Bullen, C.L., Williams, K., Fagg, C.G., Bourne, A. and Vigivon, M. (1973). Breast milk substitute: a bacteriological study. British Medical Journal, 4: 67-72.

Wimpenny, J.W.T., Kinniment, S.L. and Scourfield, M.A. (1993). The physiology and biochemsitry of biofilms. In Microbial Biofilms: Formation and Control. (eds. Deayer, S.P., Gorman, S.P. and Sussman, M.), Blackwell Scientific Publications, pp. 53-94.

World Health Organization. (1981) International code of marketing of breast milk substitute: WHO.

Wolf, J and Barber, (1968) The genus *Bacillus*: aids to the identification of the species. In. Identification methods for microbiologists, part B. pp93-109. B.M. Gibbs and D.A. Sharpton (ed) Academic Press. London.

Wong, H.C. and Chen, Y-L. (1988). Effects of lactic acid bacteria and organic acids on growth and germination of *Bacillus cereus*. *Applied and Environmental Microbiology*, 154:9: 2179-2184.

Wong, H.C. and Chen, Y.L. (1988). Growth, germination and toxigenic activity of *Bacillus cereus* in milk products. *Journal of Food Protection.* 51:9: 707-710.

Wong, H.-C., Chang, M.-H. and Fan, J.Y. (1988). Incidence and characterisation of *Bacillus cereus* isolates contaminating dairy products. *Applied Environmental Microbiology*, 54:3: 699-702.

Woolfson, A.D. (1993). The statistical evaluation of adherence assays. In Microbial Biofilms: Formation and Control. (eds. Denyer, S.P., Gorman, S.P. and Sussman, M.), Blackwell Scientific Publications, pp. 315-325.

Wuenscher, M.D., Kohler, S., Bubert, A., Gerike, U. and Goebel, W. (1993). The IAP gene of *Listeria monocytogenes* is essential for cell viability, and its gene product p60 has bacteriolytic activity. *Journal of Bacteriology*, 175:11: 3491-3501.

Zahner, V., Momen, H., Salles, C.A., and Rabinovitch, L. (1989) A comparative study of enzyme variation in *Bacillus cereus* and *Bacillus thuringiensis*. *Journal of Applied Bacteriology*. 67:275-282.

Zahner, V., Rabinovitch, L., Cavados, C.F.G. and Momen, H. (1993). Multilocus enzyme electrophoresis on agarose gel as an acid to the identification of entomapathogenic *Bacillis sphaericus* strains. *Journal of Applied Bacteriology*, 76: 327-335.

Zollner, E. and Carlier, N.D. (1993). Breast-feeding and weaning practices in Verda, 1990. Samj, 83: 580-583.