STRATHCLYDE INSTITUTE OF PHARMACY & BIOMEDICAL SCIENCES

# Development of Highly Specific Monoclonal Antibodies to *Listeria* sp. and *Listeria monocytogenes*

Kenneth Richardson BSc. (Hons)

A thesis submitted for the degree of
Master of Philosophy in Biomedical Science

University of Strathclyde
Strathclyde Institute of Pharmacy and Biomedical Sciences
2013

## Author's Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.



**Signed**

**Printed name**

**Date**

**Acknowledgements**

I would like to thank my work colleagues, especially David Higgins. I would also like to thank Dr. Dino Rotondo and John Connolly, for their support and for agreeing to be my supervisors.

And I would like to thank my parents, for always being behind me, even when my future didn't look so promising. I would like to dedicate this thesis to my grandmother Marion, who passed away peacefully among family while this was being written.

Foremost I give my most profound gratitude to Professor William H. Stimson, whom I have come to see as my mentor. Many years ago, a heavily sweating and hopelessly unemployable Chemistry graduate only just managed to find his office; whereupon said graduate was given a chance that ultimately culminated in a productive (and hopefully long and successful) career, and this thesis. Without Bill kindly giving me that opportunity to prove myself, I doubt very much if I would ever have become but a shadow of the man I am today.

**Abbreviations**

| | |
|---|---|
| Ab | Antibody |
| ActA | Actin assembly-inducing protein |
| ALOA | Agar *Listeria* (Ottaviani and Agosti) |
| AOAC | Association of Analytical Communities |
| ATCC | American Type Culture Collection |
| BCCM | Belgian Co-ordinated Collections of Micro-organisms |
| BHI | Brain Heart Infusion |
| BHIA | Brain Heart Infusion Agar |
| BLEB | Buffered *Listeria* Enrichment Broth |
| BSA | Bovine Serum Albumin |
| CFU | Colony Forming Unit |
| CLISA | Chemilumiscent Immunosorbent Assay |
| CTAC | Cetyltrimethyl ammonium chloride |
| dPBS | Dulbecco's Phosphate Buffered Saline |
| DMSO | Dymethyl sulfoxide |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EU | European Union |
| FBS | Foetal Bovine Serum |
| HAT | Hypoxanthine-Aminopterin Thymidine |
| HGPRT | Hypoxanthine-Guanine Phosphoribosyltransferase |
| HT | Hypoxanthine Thymidine |
| HRP | Horseradish Peroxidase |
| IEF | Isoelectric Focusing |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| Inl | Internalins (*Listeria* pathogenicity protein family) |
| ISO | International Standards Organisation |
| KPL | Kirkegaard and Perry Laboratories (Incorporated) |
| LLO | Listeriolysin O |
| LMG | Laboratorium voor Microbiologie, Universiteit Gent |
| *L. mono* | *Listeria monocytogenes* |

| | |
|---|---|
| mAb | Monoclonal Antibody |
| MWCO | Molecular Weight Cutoff |
| PALCAM | Polymyxin-Acriflavine-LiCl-Ceftazidime-Aesculin-Mannitol Agar |
| PAGE | Poly-Acrylamide Gel Electrophoresis |
| pAb | Polyclonal Antibody |
| PBS | Phosphate Buffered Saline |
| PBS-T | Phosphate Buffered Saline + 0.05% Tween 20 |
| PCA | Plate Count Agar |
| PCR | Polymerase Chain Reaction |
| PlcB | Phospholipase B (lecithinase) |
| OCLA | Oxoid Chromogenic *Listeria* Agar |
| RLUs | Relative Light Units |
| RPM | Revolutions per Minute |
| RPMI | Roswell Park Memorial Institute (medium) |
| RTE | Ready to Eat (pre-cooked foods) |
| SDS | Sodium Dodecyl Sulfate |
| SNBTS | Scottish National Blood Transfusion Service |
| TSB | Tryptone Soya Broth |
| YE | Yeast Extract |

**Contents** **Page**

**Figures and Tables** **Page**

**Abstract**

The aim of this study was to develop monoclonal and polyclonal antibodies against *Listeria* spp., a selective culture broth, and an immunoassay to enable a 21 hour assay to detect *Listeria* spp. in foodstuffs. *Listeria* is a common contaminant of food production. Its hardiness and ability to grow under refrigeration make it a significant contaminant of pre-cooked foods and processed dairy products. *Listeria monocytogenes* is considered the major pathogen and is responsible for the condition listeriosis, a significant public health risk with a high mortality rate. Commercial assays currently in existence are either PCR-based more than 24 hours.

A mix of species and serotypes of *Listeria* grown at 37°C and heat attenuated of was used as an immunogen. Monoclonal antibody producing cell lines were produced by hybridoma fusion. A culture broth was developed simultaneously and was able to suppress growth of endogenous food bacteria while at the same time not significantly slowing the growth of *Listeria* spp. Polyclonal sheep antibodies were purified using immunogen immobilised onto agarose.

In conclusion, the production of specific monoclonal antibodies against attenuated *Listeria* grown at 37°C was shown to be feasible. Two monoclonal lines were produced. One line was unstable, but detected 9 out of 10 of the *Listeria* strains. None of the positive cell wells in all screening plates was able to detect all *Listeria* strains simultaneously. Immunopurification of immunised sheep serum provided highly specific polyclonal antibodies which may be potentially of more interest than monoclonal antibodies for assay development, although high non-specific binding was problematic when used as both capture and detection antibody in a sandwich assay. Overall, the combination of selective broth and antibodies developed in this study were able to detect a very small inoculum (<100 CFU) of *Listeria* in all foodstuffs tested in a 21 hour assay.

# Chapter 1: Introduction

## 1.1 Genus *Listeria*

*Listeria* is a widespread bacterial genus. Consisting of gram-positive bacilli, *Listeria* was first described in 1926 by Murray, Webb and Swann[1]. It is commonly found in the environment and particularly in soil, groundwater and silage, as well as animal and human waste[2]. There are currently six described species: *L. monocytogenes, L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri,* and *L. grayi*[3].

## 1.2 *Listeria* growth characteristics

*Listeria* has a dual nature as both a parasitical pathogen and a very hardy saprophyte which makes it a problem organism that is persistent in food processing environments. It is remarkably resilient for a bacterium that cannot form spores. It can adhere as biofilms to work surfaces and can be difficult to remove even with detergents[4]. *Listeria* is a facultative psychrotroph meaning that control by refrigeration is not effective as it is capable of multiplying, albeit slowly, at these low temperatures. It is capable of surviving a wide range of temperature treatments (-1.5 to 50°C), and has been documented growing from 1 to 45°C. It can proliferate at pH from 4.4 to 9.6[5], in high salt concentrations (up to 3.5% NaCl) and can withstand both freezing and drying[6]. It is also capable of both aerobic and anaerobic respiration.

It is common for additional problem organisms (such as *Staphyloccocus* and *Bacillus*) to be found in most foods. Competing psychrotrophs (i.e. *Micrococcus*, *Aeromonas*, *Pseudomonas*) are also ubiquitous in food processing. These organisms could be present in chilled foods in amounts great enough simply to out-compete *Listeria* for nutrients when cultured in broth. Ideally, proliferation of competing organisms will be retarded or prevented by selective components in the enrichment culture medium, while growth of *Listeria* will be affected as little as possible. Bacteria species found in food are known to cause active inhibition of the growth of *Listeria*, for example *Yersinia enterocolitica*[7]. If a culture broth were not capable of repressing the growth

of these inhibitory organisms, any assay system based upon it would be prone to false negative results.

### 1.3 Pathology of *L. monocytogenes*

*Listeria monocytogenes* is considered the major pathogen and is responsible for the condition listeriosis. It constitutes a significant public health risk. The frequency of listeriosis reports are increasing for reasons that are currently unknown. In the US alone, there are an average of 2500 annual cases. Of these, 20% die of the condition[8]. Impaired or suppressed cell-mediated immunity is the most important factor in mortality[9]. Larger outbreaks of listeriosis are intermittently reported, associated with contaminated batches of factory-issued food. *Listeria ivanovii* causes listeriosis in livestock but is rare to be implicated in human listeriosis[10]. Other *Listeria* species have been reported as causing bacteraemia but it is very rare[11,12].

Although *L. monocytogenes* is a common food contaminant and is asymptomatically present in the digestive tracts of 5%-10% of the human population[13], listeriosis is far less common than other forms of food poisoning and primarily a disease of immunocompromised patients, the elderly or expectant mothers[14].

Compared to other food-borne illness, listeriosis is uncommon due to the relatively low infectivity of *L. monocytogenes*. However, listeriosis has a fatality rate which is many times greater than other food pathogens: estimates range from 20% – 25% with medical treatment; for untreated typhoid it is less than 10%[15]. The high mortality of listeriosis is directly attributable to the ability of *L. monocytogenes* to infect brain and central nervous system tissue.

*L. monocytogenes* bacilli can infiltrate the bloodstream once the organism is able to enter the host monocytes, macrophages, or polymorphonuclear leukocytes. By invading phagocyte cells it can gain easy access to the brain and in pregnant women can undergo trans-placental migration to the foetus. Listeric meningitis is the main cause of death in humans and human neonates with listeriosis. Approximately 30% of reported listeriosis infections are in pregnant women, with infection spreading to the

foetus and causing miscarriage in 20-40% of cases[16]. Pregnant women are usually advised to avoid the food products that favour the possibility of being exposed to *L. monocytogenes* such as refrigerated, ready to eat (RTE) foods: pre-cooked cold meats, non-pasteurised milk, milk products such as cheese and ice cream, raw vegetables and fish[17].

The severity of listeriosis is rated by how invasive the infection; this may depend on the health of the host, strain of *L. monocytogenes*, or infective dose. In non-invasive listeriosis, the bacteria remains restricted to the gastro-intestinal tract and the symptoms are that of mild food poisoning: flu-like symptoms, fever, gastroenteritis[18]. These symptoms normally resolve without intervention. In invasive listeriosis, sufficient numbers of *L. monocytogenes* producing virulence are able to escape the primary host defences and penetrate the cells forming wall of the gut and hence into the blood, becoming an intracellular parasite rather than a general bacterial blood infection like bactericaemia.

The pathogenicity of *L. monocytogenes* is due to complex pathogenic and virulence factors expressed by the bacterium. Invasion of host cells is promoted by bacterial cell surface proteins called internalins (Inl) which bind to mammalian cell surface factors. A complex chain of reactions then causes the cell to internalise the bacterium[19]. The first target for this internalisation is intestinal epithelial cells. From there, the bacteria can enter the bloodstream and the liver.

Phagocytosis, normally the primary cell-mediated response, actually works in favour of the infection. Once engulfed by a phagocyte, *L. monocytogenes* can escape the phagocytic vacuole before fusion with a lyzosome. It does this by excreting a phospholipase virulence factor, a lecithinase enzyme known as phospholipase B (PlcB). The bacterium is then capable of movement within the cell cytoplasm, explosive propulsion from "actin rockets" formed by polymerisation of actin via an excreted enzyme called ActA. It is even capable of penetrating the cell membrane and into other cells in physical contact with the original cell. This suite of pathogenic factors allows it to infect other cells without becoming extracellular again[20]. This infection of host cell cytosol affords it protection from the immune system[21] and allows it to spread from intestinal epithelial cells to the blood to the liver.

In healthy individuals part of the cell-mediated immunity response would normally destroy the infected cells in the liver via the action of cytotoxic CD8+ and CD4+ T-cells[22]. If the immune response is insufficient or is overwhelmed, the bacilli proliferate within the liver and infect yet more phagocytic cells that attempt, unsuccessfully, to eliminate the infection. Internalisation within these highly mobile phagocyte cells allows *L. monocytogenes* access to the brain where it can cause very serious and potentially fatal meningitis[23]. Pregnancy in placental mammals, by its nature, depresses cell-mediated immunity[24].

## 1.4 Screening methods currently available for *Listeria* in food

Mainly because of the considerable risk of listeriosis to otherwise healthy pregnant women, large scale *Listeria* screening of food is increasingly being taken up around the world. Commission Regulation (EC) No 2073/2005 (the EU) restricts foods to less than 100 CFU/g. The FDA (US) regulation is similar, but more flexible in what foods are permitted to have more than 100 CFU/g, depending on their ability to support *Listeria* growth.

### 1.4.1 Agar methods for detecting *Listeria*

The current FDA method[25] for screening involves a brief pre-enrichment of foods in rich and non-selective Buffered *Listeria* Enrichment Broth (BLEB) at 30°C for 4 hours, followed by addition of selective agents to the growth broth (acriflavine, sodium nalidixate, optionally cycloheximide for fungal control). Incubation for selective enrichment is continued at 30°C for a total of 48 hours. The enrichment culture is streaked at 24 and 48 hours on one or more of the prescribed differential selective-agars (i.e. Oxford, PALCAM, OCLA, ALOA, Rapid L mono) in order to isolate *Listeria* species. ELISA or PCR is only recommended to confirm if these isolates are *L. monocytogenes*. The ISO protocol (method 11290) is very similar. At four days from test to result and with a great amount of manual work, clearly these methods are of little use for foods that require a rapid turnaround to prevent spoilage.

### 1.4.2 PCR detection of *Listeria*

Recently, more rapid tests have been validated by AOAC. These are divided mainly between ELISA and PCR. The latter selectively amplifies *Listeria* DNA present in the food sample to find and identify specific sequences. For instance, Du Pont's BAX System *Listeria* assay targets an approximately 400 bp *Listeria*-specific genomic region. According to Du Pont, this system has been assessed to have over 98% specificity to *Listeria*, and can detect $10^5$ colony forming units (CFU) per ml *Listeria* after 24 hours enrichment. However, the sequence is not a virulence gene cluster and is also known to be found in the ubiquitous but harmless *L. innocua*[26] which can easily outgrow and mask the presence of *L. monocytogenes* when cultured together with it. Other PCR-based systems may target other *L. monocytogenes* sequences such as virulence genes, but these have also been documented in non-pathogenic *Listeria* species which may not being actively expressing pathogenic proteins[27]. The choice of a correct gene sequence both specific to the target and not found elsewhere is clearly essential for accurate screening. Furthermore, the labour and material intensive nature of PCR itself has proven disadvantageous on industrial scales. This necessitates specialised equipment and well-paid staff trained to a moderately high standard, even where self-contained kits are used. The materials are relatively expensive, and the specialised equipment (particularly automated thermal cyclers) can be prohibitively expensive. Much manipulation is necessary for a typical commercial *Listeria* PCR test kit[28]. Cultured samples must be centrifuged and washed to remove possible PCR-inhibitory compounds from foods, many of which are unknown, and many of which (such as lipids in milk) cannot be removed economically, making the test useless for certain foods. Even common broths such as Brain Heart Infusion broth (BHI) can inhibit the reaction and must be excluded from pre-enrichment design when using this method[29]. The washed cells must then be lysed with a cocktail of specialist detergents and enzymes such as lysozyme, RNase and proteinase K, which again can be inhibited by residue of the foods. Before polymerase amplification the DNA must be extracted by one of several known processes, which again can be difficult and require many reagents. This is the most critical step and that which requires the most staff training as the quality of the extraction will ultimately determine the overall assay robustness.

Poor extraction at this stage will result in failure of the PCR reaction and false negatives, regardless of all other steps.

### 1.4.3 Immunoassay detection as an alternative to PCR

Immunological detection of *Listeria* spp. is potentially as sensitive and specific as PCR, or even more so, as long as care is taken in the choice of target antigen(s) and the antibody is selective. In contrast to PCR-based systems, immunological detection – typically ELISA – is long proven to be cheap to run and automate on mass scale. ELISA is normally less affected by inhibitory matrix effects in foods than PCR[30]. Clean-up steps are unnecessary in most foods: "sandwich" ELISA have been employed to "capture" the target antigen from food via an immobilised antibody bound to microtitre plates. This allows potential matrix compounds to be washed away before the application of a detection step that may be more vulnerable to interference. ELISA may therefore require little or no manipulation of samples other than placing them on a microtitre plate, depending on the food and the assay format. This makes the tests more suitable for rapid throughput with little skilled supervision. ELISA assays are quantified by light absorbance measurement of chromogenic substrates. Chemiluminscent immunological tests promise an even more rapid and inexpensive modification: such a test can be easily added within the infrastructure of a laboratory or test facility already conducting ELISA-based food testing.

### 1.4.4 The targeting of antigens in detection

Most current commercial ELISA-based *Listeria* tests target flagellar antigens. Strains of *Listeria* are divided into serotypes based on variations of somatic (O), and surface (H) antigens (including flagella). There are currently thirteen serotypes of *L. monocytogenes* known, but others may appear at any time. Most (98%) of human listeriosis cases are caused by serotypes 1/2a, 1/2b and 4b[31], but as this varies greatly with locale and source, it cannot be assumed to always be the case. In food testing, antibody recognition of *Listeria* by H antigen is of relatively limited value. Although not all strains of *L. monocytogenes* are found to be virulent[32], the factors that make

them so are poorly understood. More importantly, a food testing environment, for safety and hygiene reasons, requires that samples be robustly heat treated before handling and assay. Most H and many O antigens associated with serotype are heat labile and would, therefore, be mostly destroyed in the process.

Another important argument against using flagellar antigen recognition is that pathogenicity of *L. monocytogenes* is thermoregulated. At 37°C the vegetative bacteria switches, via a poorly understood process, from being saprophytic to actively pathogenic, greatly altering its immunological and biochemical profile[33]. Motility, which is most pronounced at 20°C, becomes practically non-existent as it loses its flagella upon transfer to higher temperature[34]. This may be an adaptation to avoid the highly immunogenic flagella triggering immune response while in a host. *Listeria monocytogenes* also expresses virulence proteins, allowing it to invade host cells. The reduced growth at these lower temperatures necessitating expression of flagellar antigens greatly increases the length of any "enrichment" step meant to increase numbers before assay, the main consequence of which is that fitting enrichment and assay into a 24 hour window becomes unfeasible.

## 1.5 Development and selection of monoclonal antibodies for immunoassay

A monoclonal antibody is homogeneous and monospecific to a single epitope, or antigenic determinant. There may be limited cross-reactivity possible toward similar variations of the epitope structure and/or conformation, called paratopes. The epitope is the original antigenic component of the immunogen presented to a T-cell as part of the immune response following immunisation.

The ideal target for the immunological detection of *Listeria* spp. would naturally be a determinant (whether protein, carbohydrate or other) that is present in all species, serotypes and strains of *Listeria* but also not present in any other organism. Similarly, the ideal epitope target for *L. monocytogenes* would be present in only *L. monocytogenes*. This could, for example, be a virulence factor. There are many virulence factors of *L. monocytogenes* known, but their presence in any particular

strain is not always guaranteed, nor can it be assumed that their presence in other *Listeria* species would be necessarily excluded.

In order to develop monoclonal antibodies for use in *Listeria* detection assays, it is logical to assume that whatever conditions *Listeria* bacteria will be exposed to in the process of the assay protocol should also be reproduced upon the *Listeria* used for animal immunisation. This may make it more likely that antibodies will be raised against cell components that are present after culture of the *Listeria* under assay conditions. In the case of industry testing, this means bacteria grown in selective media with food, and killed by boiling.

### 1.5.1 Hybridoma fusion

In order to produce monoclonal antibody secreting cell lines, a standard hybridoma fusion approach[35] was be followed, in which spleen B-cells are fused with myeloma cells (malignant B-cells that reproduce indefinitely). In this technique, mice are immunised with the target antigen until serum samples contain a sufficient antibody titre specific to the antigen. The mouse myeloma line selected for the process is very specific: it must not secrete antibody themselves and must lack the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) enzyme gene HGPRT1. The immunised mouse spleens are removed and manually disrupted to release B-cells. The myeloma and B-cells are then chemically fused, and their nuclei and genome fused, to produce a population of genetically hybridised, immortalised cells which may secrete antibody.

Myeloma cells that did not fuse or fused incompletely have the growth advantages of being nominally genetically stable and not wasting energy excreting antibody, and would quickly overrun the hybridoma cells making them very difficult to isolate. This is avoided by the addition of aminopterin, a compound that blocks the de novo nucleotide synthesis pathway by inhibiting the enzyme dihydrofolate reductase. In the presence of thymidine and hypoxanthine, mammalian cells may instead synthesise nucleotides using the purine salvage pathway, via the HGPRT enzyme. The myeloma cells are missing the gene to encode this enzyme as stated above. Without either

nucleotide synthesis pathway active, continuation of basic life processes becomes impossible and the cells will die within 48 hours. Successfully fused cells – hybridoma cells – inherit the HGPRT1 gene from the parent B-cell and so can survive by producing nucleotides by the purine salvage pathway in lieu of the aminopterin-blocked de novo pathway. Aminopterin can be gradually withdrawn to leave only hybridoma and spleen cells, the latter of which will always naturally die over the course of a few days.

The product of this first stage of a hybridoma fusion is a highly diverse mixture of cells that may potentially secrete antibodies to any antigen the progenitor mouse has been exposed to over its lifetime. Immunised animals have a far greater titre of antibody toward the immunogen so antibodies specific to this immunogen are correspondingly more likely to be found expressed by a greater number of hybridised cells. Positive cell mixtures are subdivided and pools selected by indirect ELISA screening against the target antigen. Cell groups which do not secrete Ab specific to the target antigen are discarded in the first round of screening. These groups containing productive cells are then dilution cloned and the resultant monoclonals screened in the same way.

## 1.6 Aims of this study

The main aim of this study was to produce genus selective mAbs which recognise all *Listeria* species, and from these to isolate species selective mAbs which recognise *L. monocytogenes* alone, if present. This was attempted by immunisation with a mixture of heat-lysed *Listeria* cell components and heat-denatured in a process that would make the cultured food safe for industrial testing. All *Listeria* culture was maintained at 37°C rather than the lower temperatures used in many commercial assays and the FDA method; this was to partly prevent the expression of flagellar antigens and cause the bacteria to express the full range of virulence factors, especially in the case of *L. monocytogenes*. More importantly, this temperature is optimal for the grown of *Listeria*, minimising culture time for any test that involves them.

Monoclonal antibodies raised were intended to be for use in a rapid, sensitive and accurate detection assay for food testing. Thus any anti-*Listeria* spp. antibody was selected to be minimally cross-reactive against any other genus of bacteria, including closely related ones and those commonly found in foods in which *Listeria* is also found. A *L. monocytogenes* specific antibody is intended to recognise all *L. monocytogenes*, not only currently clinically significant strains and serotypes (this anticipates regulations that do not differentiate between avirulent and virulent types). It is also hoped that the mAb would be able to detect newly emergent pathogenic strains.

As a means of testing the effectiveness of these monoclonal lines in a food test, the co-aims of this study were to simultaneously identify culture conditions which selectively promote growth of *L. monocytogenes* whilst inhibiting potentially confounding organisms; to develop a polyclonal antibody and investigate it as sandwich assay partner to any monoclonals produced; and to apply the antibodies and broths developed in a potentially automatable ELISA test which can be used to detect the presence of pathogenic *L. monocytogenes* in the widest possible range of foods, with a minimum number of selective agents in growth media, in the shortest incubation time, in a combined pre-enrichment and immunoassay system sensitive to 1 CFU in 25 g of the original food sample.

# Chapter 2: Materials and methods

## 2.1 List of all materials and equipment

### 2.1.1 Buffers and broths

**dPBS and PBS-T**

Dulbecco's Phosphate Buffered Saline (dPBS) at 10 mM phosphate (sodium phosphate and potassium phosphate) and 135 mM sodium chloride. pH was adjusted to 7.4 with hydrochloric acid or sodium hydroxide as necessary.

PBS-T wash buffer for ELISA and CLISA was dPBS with 0.05% (v/v) Tween-20 (Sigma, P1379).

**Selective *Listeria* Broth (SLB)**

SLB was composed of 40 g $L^{-1}$ Tryptone Soya Broth (Oxoid, CM0129), 5 g $L^{-1}$ yeast extract (Oxoid, LP0021), 2 g $L^{-1}$ Lithium chloride (Fisher, L/2200/48), 1 g $L^{-1}$ Ammonium iron (III) citrate (Sigma, F5879), and 5 mg $L^{-1}$ nalidixic acid sodium salt (Sigma, N4382). Nalidixic acid was weighed out at 50 mg and dissolved in 1 ml of distilled water before being filter-sterilised through a 0.22 µm PES syringe filter to form a nalidixic acid stock, and 100 µl of this added to the broth. The broth was then autoclaved at 121°C for 15 minutes.

**Modified Selective *Listeria* Broth (mSLB)**

mSLB was prepared as SLB but for the addition of two other components. Acriflavine hydrochloride (Sigma, A8251) was added by making a stock solution and filtering for stability as with nalidixic acid sodium salt described above, with the final concentration in the broth at 1.5 mg $L^{-1}$. Nitrofurantoin (Sigma, N7878) was weighed out and directly added to the broth as a dry powder, as it was not soluble in water (final concentration 12 mg $L^{-1}$). After autoclaving the broth for 121°C for 15 minutes, it was cooled to 45°C and made to 2 mg $L^{-1}$ of ceftazidime hydrate (Sigma, C3809), again from a filtered stock solution of 20 mg $ml^{-1}$. Futher modification of the broth called for 7 mg $L^{-1}$ of cefotetan (Apollo Scientific, BIC0104) to be added after autoclaving.

**Miscellaneous Bacteria Broths and Agars**

| | | |
|---|---|---|
| Brain Heart Infusion broth (BHI) | - | Oxoid, CM1136 |
| Brilliance *Listeria* Agar Base (OCLA) | - | Oxoid, CM1080 |
| Brilliance *Listeria* selective supplement | - | Oxoid, SR0227 |
| Brilliance *Listeria* differential supplement | - | Oxoid, SR0228 |
| Rapid L mono agar | - | Bio-Rad, 356-3694 |
| Columbia blood agar base | - | Oxoid, CM0055 |
| Sheep blood (defibrinated) | - | Fisher, SR0051 |
| Cetrimide agar base | - | Oxoid, CM0579 |

Sheep blood agar was made by autoclaving reconstituted blood agar or BHI agar, cooling to 45°C in a water bath, and adding 10% defibrinated sheep blood. It was poured into plates to immediately and never re-heated.

Brilliance *Listeria* Agar was made by adding 33.6 g of Brilliance *Listeria* Agar base to 480 ml of $dH_2O$ and autoclaving at 121°C for 15 minutes. If supplementation was required, it was then cooled to 45°C in a water bath; selective supplement was reconstituted in 10 ml of 50% ethanol in sterile distilled water. Differential supplement was added directly.

**Acridinium Trigger Solutions**

Dilute nitric acid stock was made by adding 630 µl of 70% (v/v) $HNO_3$ to 99.37 ml of distilled water. Trigger solution A was made by adding 165 µl of 30% (v/v) $H_2O_2$ to 9.835 ml of solution A stock. Trigger solution A was made fresh each time.

Trigger solution B was made by dissolving 0.75g of CTAC (cetyltrimethylammonium chloride, also known as hexadecyltrimethylammonium chloride; Acros, 411410250) and 1 g of NaOH in 100 ml of distilled water. Storage was at room temperature.

**TMB Substrate**

TMB stock was made by dissolving 6 mg ml-1 3,3′,5,5′-Tetramethylbenzidine (Sigma, T2885) in dimethyl sulfoxide. This was pipetted into 250 µl aliquots in polypropylene microcentrifuge tubes and stored in the dark at -20°C.

TMB substrate was made immediately before use. 250 µl of TMB stock was added to 25 ml of 0.1 M acetate-citrate buffer pH 5.5 + 5 µl 30% (v/v) $H_2O_2$.

**Miscellaneous Reagents**

| | | |
|---|---|---|
| TMB ELISA Peroxidase Substrate | - | Interchim, UP664782 |
| Bradford Reagent | - | Sigma, B6916 |
| Cyanogen bromide activated Sepharose 4b | - | Sigma, C9142 |
| Gram staining kit | - | Sigma, 77730 |
| Casein sodium salt from bovine milk | - | Sigma, C8654 |
| Microgen *Listeria* latex confirmation assay | - | LabM, F48 |

D-(+) trehalose dihydrate was manufactured by Cargill and Hayashibara Company Ltd., Japan and purchased from web store www.trehalose.co.uk.

### 2.1.2 Commercial antibodies

KPL BacTrace Anti-*Listeria* Antibody, High Sensitivity - Kirkegaard & Perry Laboratories, 01-90-95

| | | |
|---|---|---|
| Goat anti-mouse (H+L) HRP | - | Pierce, 0031340 |
| Goat anti-mouse (γ-chain specific) HRP | - | Sigma, M1397 |
| Anti-mouse IgM (µ-chain specific) HRP | - | Sigma, A8786 |
| Rabbit anti-goat (whole molecule) HRP | - | Sigma, A5420 |

### 2.1.3 Tissue culture media

SP2/0 myeloma cell line was supplied by the University of Strathclyde.

| | | |
|---|---|---|
| RPMI medium, w/o L-glutamine, with phenol red | - | Lonza, 12-167F |
| Foetal Bovine Serum | - | Lonza, DE14-701F |
| Ultraglutamine I 200 mM | - | Lonza, BE17-605E/U1 |
| Trypan blue 0.4% solution | - | Sigma, T8154 |
| HAT supplement 50x Hybri-Max | - | Sigma, H0262 |
| HT supplement 50x Hybri-Max | - | Sigma, H0137 |
| Poly ethylene glycol, 50%, m.w. 1450, Hybri-Max | - | Sigma, P7181 |
| DMSO Hybri-Max | - | Sigma, D2650 |
| Penicillin/Streptomycin (10,000 units/ml penicillin, 10 mg/ml streptomycin) | - | Sigma, P7539 |

Foetal bovine serum was heat inactivated by placing in a water bath at 56°C for 30 minutes, before being frozen in 55 ml aliquots at -80°C. Pen-strep was stored at 4°C for a maximum of 3 months. Ultraglutamine I was stored at room temperature.

To make complete RPMI medium (10% FCS), 10 ml ultraglutamine solution, 5 ml of pen-strep solution, and 55 ml of thawed (heat inactivated) foetal bovine serum were aseptically added to 500 ml RPMI medium. This was then incubated at 37°C for at least 24 hours to ensure sterility. Turbid solutions or abnormally yellow (acidic) solutions were discarded. Media was stored at 37°C for up to 2 weeks, or up to 3 months at 4°C.

To make cryopreservation Medium (90% FBS), 5 ml of sterile DMSO was added to aseptically to 45 ml of FBS. This was then stored at 4°C.

### 2.1.4 General equipment

Berthold LB96 microplate luminometer (with WinGlow software)
Cecil CE 292 Digital Ultraviolet Spectrophotomer
Grant SBB Aqua 12 Plus boiling water bath
Thermo Scientific Multiskan FC absorbance microplate reader
Tecan M16/4R Columbus Plus microplate washer
Thermo Scientific Heraeus Megafuge 16R Centrifuge
Seward Stomacher 400
"Mr. Frosty" cryogenic freezing containers
Bright Line Haemocytometer
Bio-Rad Biologic LP System
Stericup-GP filter units 0.22 μm PES (Millipore, SCGPU11RE)
Labnet VX-200 vortex mixer

## 2.2 Establishment of *Listeria* stocks

Ten *Listeria* strains were chosen to represent a broad range of *Listeria* antigens that would encompass both those presently found and if possible all future strains. Representatives of all six known *Listeria* species were picked, along with four *L. monocytogenes* serotypes including the three most clinically significant, 4b, 1/2a and 1/2b. They were purchased from BCCM/LMG (Laboratorium voor Microbiologie, Universiteit Gent) as freeze-dried cultures sealed in glass ampoules. Each strain was assigned a letter for easy reference. The ten strains chosen for are shown in Table 2.1.

Table 2.1: *Listeria* strains chosen for this study

| Ref. | *Listeria* Species | Stated Serovar | LMG Cat No. | ATCC No. |
|---|---|---|---|---|
| **A** | *L. monocytogenes* | 1 | 21263 | 19111 |
| **B** | *L. innocua* | 6a | 11387 | 33090 |
| **C** | *L. monocytogenes* | 4b | 21264 | 13932 |
| **D** | *L. welshimeri* | 6b | 11389 | 3589 |
| **E** | *L. monocytogenes* | 4a | 16783 | 19114 |
| **F** | *L. ivanovii* | 5 | 11388 | 19119 |
| **G** | *L. seeligeri* | 1/2b | 11386 | 35967 |
| **H** | *L. grayi* | Not stated | 16490 | 19120 |
| **I** | *L. monocytogenes* | 1/2b | 16780 | - |
| **J** | *L. monocytogenes* | 1/2a | 23190 | - |

Upon receipt from LMG in lyophilised form, the supplier's instructions were followed to revive the cultures: each glass ampoule was opened with a tube cutter and the preserved dry mass re-suspended in 100 ml of warm (30ºC) Brain Heart Infusion (BHI) in 250 ml glass flasks sealed with cotton wool. This was cultured for 24 hours at 30ºC with gentle (100 RPM) shaking. Subcultures were taken by streaking a 10 µl loopful of broth onto BHIA (Brain Heart Infusion Agar) plates and culturing again for 24 hours at 30ºC.

Single colonies were taken from these subculture plates and used to inoculate cryobeads for long-term storage and establishment of a reliable *Listeria* library. They were also validated as being the correct, uncontaminated organism by plating out and testing single colonies with API *Listeria* test kits (Biomerieux). Single colonies were

also taken from the selective Brilliance agar and used to inoculate a separate set of cryobeads, as a reliable backup supply.

### 2.2.1 Validation of *Listeria* stocks

From the same plates used to inoculate the cryobeads, colonies were taken and sub-cultured by streaking onto BHI-Agar supplemented with 7% (v/v) sheep blood, and onto Brilliance Chromogenic Agar (also known as Oxoid Chromogenic *Listeria* Agar, OCLA), which is moderately selective for *Listeria*. These were cultured at 37°C for 48 hours and examined visually on a light panel after this time.

The presence of the haemolysin Listeriolysin O (LLO) was determined by a zone of ß-haemolysis indicated by the yellowing of the red colour of agar around individual colonies on blood agar (not on mats of growth or on the original streak well).

*Listeria* colonies on OCLA are bright green due to glucose metabolism via an enzyme that cleaves a proprietary chromogenic substrate (X-glucoside chromogenic mix) based on glucose bound to a green chromogen. Lecithinase production, specific in *Listeria* only in *L. monocytogenes*, was identified by a cloudy halo around individual colonies. Failure of the profile of the colonies on OCLA and blood agar to match known profiles for the *Listeria* species being tested was taken to indicate contamination of the stock. In this eventuality the originator stock would have been discarded.

Finally, colonies were taken from the blood agar and sub-cultured onto BHI-Agar. Colonies from this subculture were then identified by API *Listeria* test and placed on BHI-Agar slopes and sent to the Health Protection Agency for third party serotyping.

## 2.3 Preparation of *Listeria* antigen

*Listeria* antigen prepared was a crude mixture of heat-attenuated lysed bacterial cell contents. Because this would be the mixture present in end-result of any commercial food testing assay rather than a purified form, it was used to immunise animals for raising of monoclonal cell lines and polyclonal antibody serum, and used as a positive control in all assays performed in this study.

### 2.3.1 Culture and harvesting of *Listeria*

*Listeria* cultures were initiated by adding a library cryobead of each of the ten *Listeria* strains to 50 ml of BHI and culturing for 22 hours at 37°C. This was then centrifuged at 2200 RCF for 30 minutes and the supernatant discarded. The pellet was washed and re-suspended with sterile 12 mM PBS pH 7.2. This process was repeated twice more to remove any remaining broth.

The remaining pellet was then re-suspended in 5 ml of PBS and 100 µl was removed for enumeration. The remainder was attenuated and lysed by immersing the centrifuge tube in a boiling water bath (100°C) for 20 minutes. The boiled antigens were then stored in undiluted form at -80°C.

### 2.3.2 Enumeration of *Listeria*

The 100 µl of live bacteria were set aside was then quantified by turbidity in a partly proprietary method. 600 µl of PBS was added to a disposable polystyrene cuvette (semi-micro size). Small amounts of bacterial suspension were added (1-10 µl) and mixed and absorbance read at 540 nm. Suspension or PBS would continue to be added until the absorbance was 0.900 (± 0.05). This was taken as a reference point of $10^7$ cells per ml, and although relatively precise, this figure was understood not to be literally accurate. It also represents total cells, rather than viable colony forming units (CFU). However it did provide a fast and reproducible method as a base for more accurate determinations.

The dilution necessary to provide $10^7$ cells/ml was then used to derive the concentration of the original stock. The formula for calculating the number of cells in the original stock in this way is as follows:

$$C = \frac{V_E}{Vs} \times 10^7$$

Where C is the number of cells per ml in the original stock, $V_E$ is the total volume of liquid added to the cuvette, and $V_s$ is the total volume of bacterial suspension added to the cuvette.

## 2.4 Development of sandwich CLISA

A commercial polyclonal antibody, KPL BacTrace anti-*Listeria* High Sensitivity, was purchased due to its claimed spectrum of *Listeria* detection and low cross-reactivity to other species. It was evaluated for its affinity toward the ten *Listeria* strains cultured at 37°C for 20 hours and attenuated by 20 minutes boiling. It was also investigated in sandwich immunoassay format and its ability to perform as a detection antibody when labelled with acridinium ester.

### 2.4.1 Evaluation of KPL antibody by indirect ELISA

To test the applicability of the KPL polyclonal, a 96 well ELISA plate was coated with all *Listeria* strains. *Listeria* stocks were diluted to $10^6$ cells/ml in PBS and plated out at 100 µl/well, eight wells per strain A through J and six wells containing only buffer. The plate was then incubated at 37°C for 1 hour and washed using a Tecan plate washer. Wash buffer was PBS-T: 10 mM D-PBS pH 7.4 + 0.05% (v/v) Tween 20.

The plate was then blocked by adding 300 µl of blocking buffer: 3% (w/v) casein sodium salt (in 10 mM PBS pH 7.4), and incubating for 1 hour at 37°C, after which time the plate was again washed as before.

The KPL BacTrace antibody was shipped in 1 mg lots in lyophilised form. It was re-dissolved in 1 ml 50% glycerol/0.15 M saline for a 1 mg $ml^{-1}$ stock. This was stored at -20°C. Because it did not freeze, it could be removed and replaced indefinitely in cold storage without aliquoting.

The KPL BacTrace was then diluted to 1 µg $ml^{-1}$ in PBS (1:1000) and plated out at 100 µl/well in all wells. The plate was incubated at 37°C for 1 hour and washed.

Anti-Mouse H+L HRP was supplied in lyophilised form and reconstituted according to the manufacturer's instructions: 1 ml 0.147 M saline and 1 ml glycerol were added to the vial and gently mixed until dissolved. This stock could then be stored at -20°C.

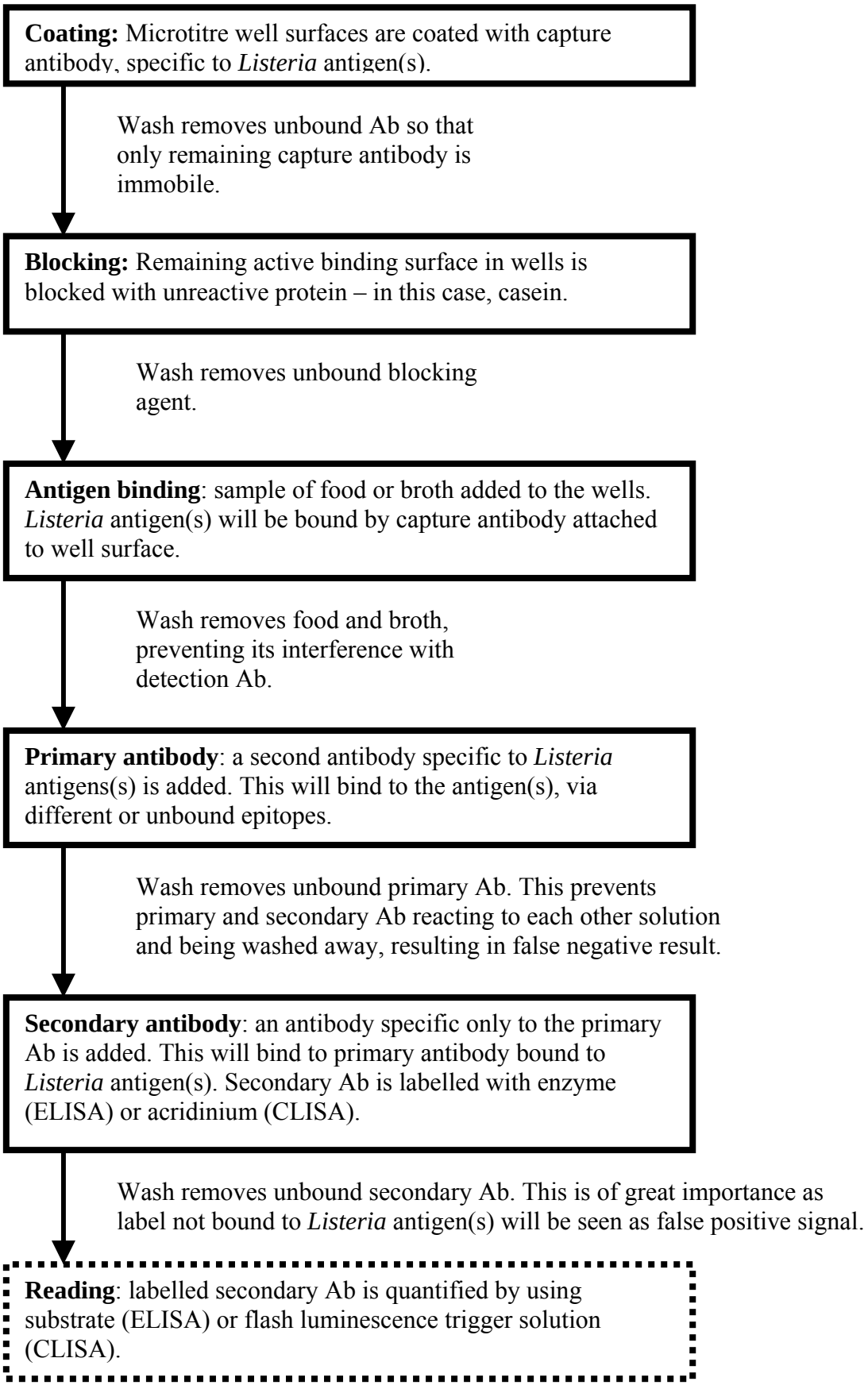
Anti-Mouse HRP stock was diluted 1:2500 in blocking buffer and plated out at 100 µl/well all wells, and incubated at 37°C for 1 hour and washed.

TMB stock was made by dissolving 3,3′,5,5′-tetramethylbenzidine (TMB) in dimethyl sulfoxide (DMSO) to the concentration of 6 mg $ml^{-1}$. This was dispensed into 250 µl aliquots and stored at -20°C.

Finally, substrate was added to the plate. 250 µl of TMB stock was diluted in 25 ml of 0.1 M acetate-citrate buffer pH 5.5 supplemented with 5 µl 30% (v/v) $H_2O_2$ and plated out at 100 µl/well all wells. Colour was allowed to develop for 20 minutes at room temperature in the dark then the reaction stopped with the addition of 100 µl 10% (v/v) $H_2SO_4$ in all wells. The absorbance of the plate was then read at 450 nm in a Titertek Multiskan MCC/340.

Figure 2.2 shows an overview of the steps involved in an indirect sandwich ELISA (or CLISA).

Figure 2.2: A simple diagram of the steps involved in indirect sandwich CLISA and ELISA



### 2.4.1 Acridinium ester labelling of KPL antibody

In order to use the KPL polyclonal Ab as a detection antibody in a CLISA assay, it first had to be labelled with acridinium. This was achieved via simple NHS-ester based reaction and removing unbound acridinium using size-exclusion chromatography.

50 µl of the antibody stock at 1 mg $ml^{-1}$ (therefore containing 50 µg antibody) was placed in a black, polypropylene screw cap reaction vial. To this was added 250 µl of labelling buffer (0.1 M phosphate buffered saline pH 8.0). 5 µl of 1 mg $ml^{-1}$ acridinium C2 NHS ester stock (therefore containing 5 µg) was added to the vial. The vial was placed on a rocking stirrer at room temperature. After 10 minutes the reaction was then stopped by the addition of 100 µl of 1% (w/v) lysine hydrochloride in labelling buffer. This was stirred for a further 20 minutes at room temperature.

A PD-10 desalting column was equilibrated with 5 column volumes (25 ml) of purification buffer – 0.1 M PBS pH 6.3 containing 0.1% (w/v) bovine serum albumin (BSA) and 0.05% (w/v) sodium azide. The contents of the reaction vial were then applied to the column and allowed to run through. A further 2.1 ml of purification buffer was added and allowed to run through. This brings the protein close to the end of the column. The column was then eluted with purification buffer; eight fractions were taken consisting of approximately 300 µl per fraction.

To test for the acridinium activity in each fraction, 2 µl of each was added to 0.5 ml of PBS, vortexed, and 5 µl of this spotted into the centre of a well on a white plate and read using a Berthold luminescence plate reader. See section 2.3.3 for full details.

The three highest fractions (normally 2, 3 and 4 or 3, 4 and 5) were pooled and stored in a black microcentrifuge tube at 4°C.

### 2.4.3 Sandwich CLISA

KPL Anti-*Listeria* was diluted to 1 μg $ml^{-1}$ in 0.1 M carbonate buffer pH 8.5 and plated into white microtitre strips at 100 μl/well. This was incubated at 37°C for 1 hour and washed using the usual method (PBS-T and plate washer), and dried.

The strips were blocked with blocking buffer containing 3% (w/v) casein (sodium salt) in D-PBS pH 7.4 at 300 μl/well incubated for 1 hour at 37°C, washed, and finally dried.

*Listeria* antigen stocks made in Section 2.2.1 were diluted to $10^6$, $10^5$, $10^4$, $5x10^3$, $10^3$ and $10^2$ prelative cells/ml in sterile SLB and plated out at a column per strain. *E. coli* and *S. enteriditis* were again used as negative controls at $10^6$/ml. The plate was incubated for 30 minutes at 37°C, washed and dried.

Acridinium-labelled KPL antibody was then diluted 1:100 in blocking buffer and plated out at 100 μl/well in all wells. The plate was then incubated for 30 minutes at 37°C, washed and dried.

The microtitre strips were then read using a Berthold LB96 plate luminometer with dual injection system. Injector P was set at 30 μl, with a 1.6 s delay and no measuring interval. Injector M was set to 60 μl, with no delay and a 1 s measurement interval.

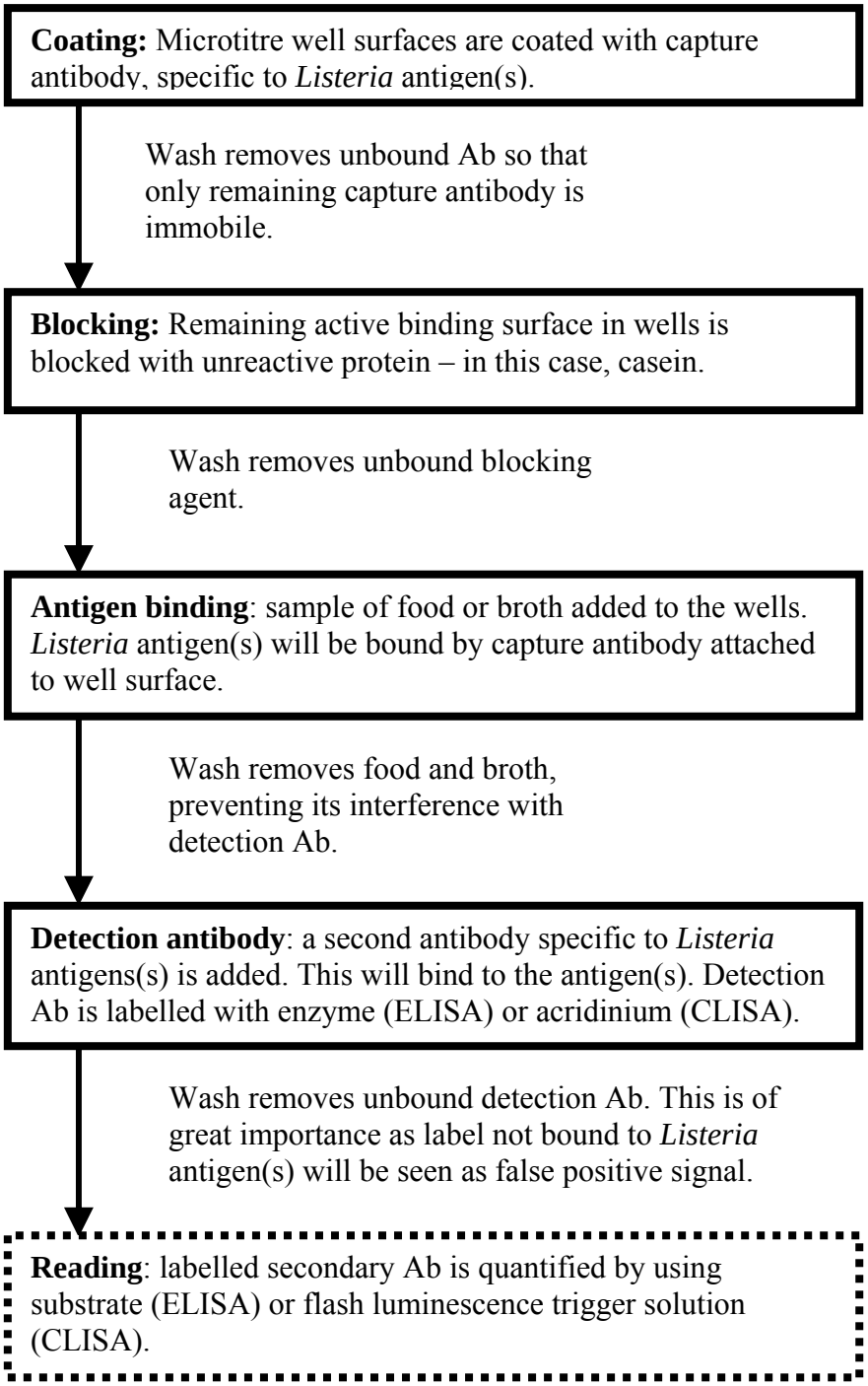
Trigger solution A stock was made by adding 630 μl of 70% (v/v) $HNO_3$ to 99.37 ml of distilled water. Complete solution A, which was made fresh before every run, was made by adding 165 μl of 30% (v/v) $H_2O_2$ to 9.835 ml of solution A stock. This was used to prime Injector P.

Trigger solution B was made by dissolving 0.75g of CTAC (cetyltrimethylammonium chloride) and 1 g of NaOH in 100 ml of distilled water. This was used to prime Injector M.

The plate reader then injected solution A into a well, waited 1.6 s, then injected solution B and immediately recorded light output for 1 s. This was repeated for every well.

Figure 2.3 shows an overview of the steps involved in a direct sandwich CLISA (or ELISA).

Figure 2.3: A simple diagram of the steps involved in direct sandwich CLISA and ELISA



## 2.5 Raising of polyclonal antibody against *Listeria*

Sheep were immunised with a mixture of *Listeria* antigens to provide a polyclonal sandwich assay counterpart to the monoclonal antibody being developed. Sheep sera were screened by indirect ELISA against attenuated *Listeria* bound to microtitre plates. Sera were screened both prior to and after immunisation.

### 2.5.1 Pre-screening of sheep for endogenous anti-*Listeria* antibody titre

Sheep bleeds were delivered by SNBTS as serum ready to use. Each sheep was identified by a number. Screening of the serum was carried out by indirect ELISA, using a rabbit anti-goat polyclonal antibody labelled with horseradish peroxidise (HRP). There proved sufficient similarity between goat and sheep IgG for detection by an anti-goat polyclonal.

Antigen from all ten validated *Listeria* strains was diluted to a relative concentration of $10^6$ cells/ml in 10 mM dPBS pH 7.4 and plated out at 100 l/well in a microtitre plate, one column per strain. *Salmonella enteritidis* and *E. coli* antigens were also used at an equivalent concentration of $10^6$ cells/ml in the two remaining columns as negative controls. The plate was incubated at 37°C for 1 hour and washed using the method in the previous section.

The plate was then blocked with 300 µl/well blocking buffer (3% (w/v) casein in 10 mM D-PBS pH 7.4), all wells. It was incubated at 37°C for 1 hour and washed and dried.

Pre-immune sera from sheep were diluted 1:1000 in 10 mM dPBS pH 7.4. It was plated out in two rows per sheep, 100 µl/well. It was incubated and washed and dried as before.

Anti-goat HRP was diluted 1:2500 in blocking buffer and plated out at 100 µl/well, all wells. It was incubated and washed and dried as before.

TMB stock was thawed and 250 µl diluted in 25 ml of 0.1 M acetate-citrate buffer pH 5.5 supplemented with 5 µl 30% (v/v) $H_2O_2$ and plated out at 100 µl/well all wells. Colour was allowed to develop for 20 minutes at room temperature in the dark then the reaction stopped with the addition of 100 µl 10% (v/v) $H_2SO_4$ in all wells. The absorbance of the plate was then read at 450 nm.

Three sheep were chosen according to low titres of *E. coli* and *S. enteridis* specific antibodies and, where evident, higher than background levels of anti-*Listeria* activity.

### 2.5.2 Immunisation of sheep with attenuated *Listeria*

The ten validated strains of *Listeria* were cultured and washed as in previous antigen preparation; however they were grown in Selective *Listeria* Broth (SLB) at 37°C as this was the media planned for the food assay. After centrifugation and washing, live bacterial pellets were quantified by turbidity measurement at 540 nm as earlier described to estimate cells/ml. Each sample was streaked onto OCLA agar and Blood Agar to validate and attenuated by immersion in a boiling water bath for 20 minutes. After having been boiled, the *Listeria* samples were ensured as non-infectious by streaking on BHI Agar. They were then stored at -80°C.

Rather than a relative number of cells per ml, sheep had to be immunised with a known quantity of protein limited to 200 µg (in 1.25 ml) per injection due to Home Office rules (resulting in 160 µg $ml^{-1}$). To quantify the protein content in the boiled *Listeria*, a Bradford assay was performed. BSA was diluted to known standards of 0.1 to 2 mg $ml^{-1}$ and 5 µl of each standard spotted into wells of a microtitre strip. Each sample was also spotted into wells at 5 µl neat, 1:2 dilution and 1:4 dilution to ensure that the value fell within the range of standards.

To read, 100 µl of Bradford reagent was added to all wells and the strips gently shaken, left for 2-3 minutes at room temperature, and read by absorbance plate reader at 570 nm. According to the documentation of the reagent, the relationship between protein concentration and Bradford reagent is always linear; the BSA standards were

fitted to a linear regression curve and the calculated formula used to derive the concentration of the samples.

Sheep were reassigned letters from their original SNBTS designations; sheep 107 was sheep A, sheep 108 sheep B, and sheep 111 sheep C. Rather than give all three sheep identical immunisations, *Listeria* strains were grouped into three: those with poor ELISA and CLISA response, those with strong response, and those with moderate response. This was to try to prevent strains that produce a strong response overwhelming those with weaker response and providing poor immunisation to those strains. Two groups were made up of three strains and the third four strains.

The 200 μg protein allowed in each 1.25 ml injection was split equally between these strains.

Fifty of these one-injection aliquots were made and sent to SNBTS who mixed each with an equal volume of Freund's Complete (for immunisations) or Freund's Incomplete (for boosters) and injected subcutaneously.

| Strain | Serovar | ATCC/LMG | | CLISA Response | ELISA Response | Sheep immunised |
|---|---|---|---|---|---|---|
| *A) L. monocytogenes* | 1 | ATCC19111 | | + | + | Sheep C |
| *B) L. innocua* | | ATCC33090 | | +++ | +++ | Sheep B |
| *C) L. monocytogenes* | 4b | ATCC13932 | | ++ | ++ | Sheep C |
| *D) L. welshimeri* | | ATCC3589 | | ++++ | ++++ | Sheep A |
| *E) L. monocytogenes* | 4a | ATCC19114 | | ++++ | ++++ | Sheep A |
| *F) L. ivanovii* | | ATCC19119 | | + | + | Sheep C |
| *G) L. seeligeri* | | ATCC35967 | | ++ | + | Sheep A |
| *H) L. grayi* | | ATCC19120 | | +++ | ++++ | Sheep A |
| *I) L. monocytogenes* | 1/2a | LMG16780 | | ++ | +++ | Sheep B |
| *J) L. monocytogenes* | 1/2b | LMG23190 | | ++ | ++ | Sheep B |

Table 2.4: Sheep immunisation pools. This shows the mixtures of *Listeria* spp. used to immunise the sheep. The "CLISA response" and "ELISA response" were judged semi-quantitively from ELISA and CLISA run using KPL BacTrace, and are relative to the response of the other *Listeria* spp. An approximate magnitude of response is denoted by the number of + symbols with + being a low reading (450 nm or RLUs) and ++++ being a very high reading.

### 2.5.3 Immunoaffinity purification of sheep polyclonals

In order to isolate pAb specific to *Listeria* antigens, it was necessary to allow them to bind to these antigens. *Listeria* antigens were immobilised by covalent binding to an agarose support. All ten strains were used, and the same stock used to immunise the sheep was employed. The stocks were pooled so that there was 1 mg of protein from each stock in the pool.

*Listeria* antigen pool was then diluted in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.4) to 10 mg total protein in 1 ml of buffer**.**

Cyanogen bromide pre-activated Sepharose 4b was supplied by Sigma in stabilised, lyophilised form. It was prepared by soaking 200 mg of dry product in 1 mM HCl at 4°C in a 50 ml polypropylene centrifuge tube for at least 30 minutes to allow it to swell and to remove lactose stabiliser. It was then spun at the same temperature in a

centrifuge at a very low speed (100 RPM) for 30 minutes (no brake) and the supernatant discarded.

The resin was again washed in this way with 30 ml of nanopure water then a further time with 30 ml coupling buffer. It was then immediately transferred to the *Listeria* antigen pool coupling buffer in a 15 ml polypropylene centrifuge tube.

Resin and antigen was mixed on an end-over-end stirrer overnight at 4°C. Unreacted antigen was washed away using coupling buffer and centrifugation as described above; the supernatant was discarded. Unreacted cyanate ester groups on the resin were then blocked with the re-suspension of the resin of 30ml 1 M ethanolamine pH 8.0. This was mixed for 2 hours at room temperature, again on the end-over-end stirrer.

The gel was then washed as above using an alternating cycle of basic and acidic wash buffers: coupling buffer at pH 8.4, then 0.1 M acetate + 0.5 M NacCl, pH 4. This cycle was repeated four more times. This step removed any blocking buffer and any unbound protein. The resin was then applied to a 10 ml column and stored 1.0 M NaCl + 0.05% sodium azide at 4°C until use.

When the column was to be used, it was equilibrated with 10 mM PBS pH 7.2. Sheep serum was pooled and 10 ml diluted with 10 ml PBS. This was then applied to the column, using a Bio Rad sdfsdfd to monitor the absorbance of the flowthrough at 280 nm. The column was washed with PBS until the A280 value had reached a stable background level. Elution buffer (0.1 M glycine-HCl, pH 2.7) was then applied and eluent collected when the A280 trace began to rise. The eluent was immediately brought to neutral pH by the addition of 1 M sodium hydrogen carbonate, then twice dialysed against PBS overnight using 12 kD MWCO dialysis tubing.

The eluted protein was quantified by absorbance at 280 nm: the absorbance reading at 280 nm was divided by 1.4 to give approximate concentration in mg, with the assumption that it is primarily IgG.

### 2.5.4 Capture CLISA with immunoaffinity purified sheep pAb

Immunoaffinity purified anti-sheep pAb was tested both for its affinity to *Listeria* antigens but also for its suitability when partnered with a second antibody in a sandwich capture. It was also assessed for cross-reactivity to other bacteria.

KPL Anti-*Listeria* was diluted to 1 μg $ml^{-1}$ in 0.1 M carbonate buffer pH 8.5 and plated into white microtitre strips at 100 μl/well. This was incubated at 37°C for 1 hour and washed using the usual method (PBS-T and plate washer), and dried.

The strips were blocked with blocking buffer containing 1% (w/v) casein and 0.25 M trehalose in D-PBS pH 7.4 at 300 μl/well and incubated for 1 hour at 37°C. The blocking buffer was aspirated and the plate dried in a Class 1 flow cabinet, but not washed. The plate was then stored in a dessicator at 4°C until use.

Fifty microlitres of the purified sheep pAb was labelled with acridinium using the protocol described in Section 2.3.2.

*Listeria* antigen stocks made in Section 2.2.1 were diluted to $10^6$, $10^5$, $10^4$, $5x10^3$, $10^3$ and $10^2$ prelative cells/ml in sterile mSLB and plated out at a column per strain.

Several other bacteria found in the food testing were tested against the antibody. These were grown in nutrient broth, but enumerated and attenuated in the same way as the *Listeria* standards.

*Kurthia zopfii* (NCTC 405)
*Erysipelothrix rhusiopathiae* (NCTC 1694)
Presumptive *Brevibacillus laterosporus* (from infant formula milk)
*Enterococcus faecalis* (NCTC 12697)
*Escherichia coli* (isolated from uncooked chicken nuggets)
*Enterobacter cloacae* (isolated from beef mince in BHI)
*Pseudomonas aeurogenosa* (NCTC 10662)
*Staphylococcus aureus* (NCTC 6871)
*Salmonella senftenberg* (NCTC 4444)
*Bacillus cereus*, from infant formula milk, identified by Microgen Bacillus ID.

These were also plated out at $10^6$ relative cells per ml, 100 ul/well. The diluent was mSLB, despite the fact that they were grown in nutrient broth. The plate was then incubated for 30 minutes at 37°C, washed and dried.

Acridinium-labelled immunopurified sheep antibody was then diluted 1:50 in blocking buffer and plated out at 100 µl/well in all wells. The plate was incubated for 30 minutes at 37°C, washed and dried.

The plate was then read by plate luminometer with trigger solutions A and B as in Section 2.3.3.

### 2.5.5 Assessment of an existing commercial *Listeria* ELISA

To provide a basis of comparison with pAb and mAb being developed, an Association Française de Normalisation (AFNOR) accredited *Listeria* ELISA kit was run on the *Listeria* stocks produced for this study. (Certificate RAY 32/03-07/10 can be obtained from RayAl Ltd or from AFNOR Certification.)

The kit used was supplied by RayAl and was developed by Bioline Reagents Ltd. Its manual specifically states that it is specific to *Listeria* flagellar antigens, and that the food to be screened for *Listeria* must be cultured at 30°C. Although the kit specifies that food being screened for *Listeria* should be cultured 24 hours in Half Fraser broth and another 24 hours in proprietary RayAl broth called RELM, this step was omitted. Instead, the ten *Listeria* strains were grown for 20 hours as in Section 2.1, but at 30°C instead of 37°C. Enumeration and boiling was done identically as before.

Pre-coated microtitre strips were supplied in the kit. Bacteria, including *Listeria* stocks grown at 30°C and 37°C, were diluted to $10^6$ relative cells per ml in SLB and plated out at 100 µl/well in duplicate. Positive and negative controls, supplied in the kit, were applied at the same volume without further dilution.

Incubation was 30 minutes at 37°C, followed by a wash. HRP-labelled detection antibody was supplied ready-to-use in the kit, and was plated out at 100 µl/well. Incubation was 30 minutes at 37°C, followed by a wash. Substrate (TMB based) was also supplied ready to use in the kit. It was pipetted into all wells. Development time was 30 minutes at room temperature, followed by 100 µl/well of supplied stop reagent and reading by plate absorbance reader, 450 nm.

### 2.5.6 Capture CLISA using immunopurified sheep pAb only

With the anti-*Listeria* specificity of the immunopurified sheep pAb confirmed, all that remained was to test its effectiveness when used as both capture antibody and detection antibody in immunoassay. Rather than run a full assay, the level of non-specific binding was assessed by running a capture assay with a range of different levels of pAb coating and acridinium-labelled pAb. *Listeria monocytogenes* serovar 4b (grown at 37°C) was used as a positive control. The background control was *Kurthia zopfii*, which the purified sheep pAb was confirmed not to bind to (Section 3.4.4).

Immunopurified pAb was diluted to 1, 2, 5, 10 and 20 μg $ml^{-1}$ in PBS and plated out (100 μl/well) in duplicate columns of a 96 well white microtitre plate. Incubation time was 1 hour at 37°C, followed by washing. Blocking was with 3% (w/v) casein in PBS, followed by the same incubation and washing.

*Listeria* and *Kurthia* stocks were diluted to $10^6$ relative cells per ml in SLB and plated out in alternating rows at 100 μl/well. After 30 minutes incubation (37°) the plate was washed.

Immunopurified sheep pAb labelled with acridinium was diluted 1:40, 1:60, 1:80 and 1:100 in 3% (w/v) casein in PBS and used at 100 μl/well. Incubation time was 30 minutes (37°C) after which the plate was read on a plate luminometer with trigger solutions A and B as before.

## 2.6 Raising of monoclonal cell lines against *Listeria*

In order to produce a monoclonal cell line, mice had to be immunised, tested regularly and finally the best responders sacrificed and B-cells from their spleens fused with myeloma cells. Because mice are cloned and kept in relatively aseptic conditions, they did not have to be pre-screened for existing titre.

### 2.6.1 Immunisation of mice with attenuated *Listeria*

The ten validated strains of *Listeria* were cultured and washed as in previous antigen preparation (section 2.4.2) and in SLB at 37°C. In this case protein content was not required to be known. After centrifugation and washing, in a Class 2 cabinet to avoid contamination by other organisms, washed bacteria pellets were quantified by turbidity measurement at 540 nm to estimate cells/ml. Each sample was attenuated by immersion in a boiling water bath for 20 minutes and diluted so that each 25 µl dose of the immunogen contained the equivalent of $10^7$ cells in one lot and $3x10^7$ in another lot, for groups of 7 and 8 female BALB/C mice respectively. 10 µl loopfuls from the immunogens were streaked onto nutrient agar and left overnight at 30°C to verify sterility.

The immunisations were carried out by SNBTS. Each 25 µl dose was mixed with an equal amount of adjuvant (Freund's Complete) and injected subcutaneously. The mice were immunised every 2 weeks and an additional booster (using Freund's Incomplete) was given 1 week before sacrifice.

### 2.6.2 Pre-immunisation screening of mouse sera for existing anti-*Listeria* titre

Mouse blood was collected by tail bleed, and left at 4°C for 20 hours to allow clotting. The clot was removed using forceps and discarded, the serum spun at 500 RCF and the supernatant drawn off by pipette.

The mouse sera were screened by indirect ELISA in a method identical to that used to screen the sheep sera with the single exception that anti-mouse IgG H+L HRP was used as the secondary antibody for detection. Coating, blocking and sera dilutions remained the same as before.

Mouse chosen for fusion had a high titre against all *Listeria* strains and as little as possible against *S. enteriditis* (NCTC 4444) and *E. coli* (NCTC 11560).

### 2.6.3 Hybridoma fusion

The mouse myeloma line SP2/0 was used a myeloma base. SP2/0 cells were supplied from liquid nitrogen storage in a cryovial. They were revived by thawing rapidly in a water bath at 37°C and adding the vial contents to a 25 $cm^2$ vented tissue culture flask 2 ml of complete RPMI which had been given 30 minutes to acclimatise in the incubator (37°C, 5% $CO_2$). The cells were fed daily over several days and expanded when necessary until at least two 75 $cm^2$ flasks were confluent with the cells.

The day before fusion, SP2/0 cells were fed to ensure cells were in log phase of growth when harvested. On the day of fusion, the SP2/0 should be healthy and 70 to 80% confluent. Flasks that were overpopulated or where cells are granular or growing slowly were rejected.

On day of fusion, SP2/0 were harvested by gentle shaking and centrifuged at 200 RCF for 7 minutes and the supernatant discarded. They were then pelleted and washed twice more in the same way with incomplete RPMI to remove all traces of serum. The clean pellet was re-suspended in 1 ml of incomplete RPMI. 60 µl of this suspension was added to 30 µl 0.4% (w/v) trypan blue solution. Viable cells were counted by adding 2-10 µl of this to a bright line haemocytometer and counting only colourless cells. The original cell concentration was then calculated by taking the average of the four haemocytometer counts and multiplying by $1.5x10^4$ to compensate for dilution due to trypan blue dilution and for haemocytometer volume.

Selected mice were killed by exsanguination while under deep anaesthesia. The spleen was isolated and washed 3 times with incomplete RPMI in a sterile Petri dish.
In a fresh Petri dish, the spleen was cut into 3-4 parts with a sterile scalpel and sections gently squeezed to release the milky lymphocyte suspension, taking care not to disrupt tissue and release fibroblasts. The cell suspension was then carefully drawn into a 1 ml syringe with narrow gauge needle (21G) to exclude large particles of fat and fibrous tissue.

Slowly in order to avoid lysis of cells, the lymphocyte suspension was added to a 50 ml centrifuge tube containing 30 ml of un-supplemented (incomplete) RPMI medium and l00 µl of this cell suspension was then added to 100 µl of 4% (v/v) acetic acid to lyse erythrocytes. The remaining cells were by counted by haemocytometer as before.

All of the spleen cells were placed in a 15 ml centrifuge tube, and four times as many myeloma cells added for a 4:1 ratio of myeloma/spleen cells. 10 ml of incomplete medium was added and the cells mixed gently to ensure an even distribution.

The cell mixture was then centrifuged at 200 x g for 5 minutes. The supernatant was discarded and care was taken to ensure the pellet was as dry as possible using a sterile polypropylene pastette if necessary. The pellet was disrupted by sharply tapping the outside of the tube until the cells were dispersed.

1 ml PEG at 37°C was added dropwise very slowly over 7 minutes, tapping the cell mix gently between each drop to facilitate mixing. When all PEG was added, cells were stood at room temperature for 2 minutes.

The PEG was then diluted out by slow dropwise addition of 20 ml RPMI 1640 over 5 minutes with the same tapping as before. The cells were stood a further 10 minutes at room temperature.

Cells were washed twice with incomplete RPMI and centrifugation as before to remove all traces of PEG. The pellet was diluted to $2.5x10^6$ cells per $ml^{-1}$ in HT medium and incubated for two hours at 37°C.

The cell suspension was then plated out at 100 µl/well in the centre 64 wells of three lidded cloning plates at three different concentrations: $2.5x10^6$/ml, $1.25x10^6$/ml, $0.6x10^6$/ml. To the outside wells on the left first plate, 100 µl/well 8 wells un-fused spleen cells were added as a control. To the outside wells on the left, 100 µl/well unused SP2/0 were added as a second control. Sterile PBS was added to the outside wells for equal heat and humidity distribution. 150 µl of HAT medium was added to all wells containing cells and the plates incubated in 100% humidity, 37°C and 5% $CO_2$.

After 7 days the media was refreshed and cells fed by aspirating off 100 µl and replaced with 100 µl fresh HAT medium. Feeding was repeated 3rd or 4th day. At this point, when all of the myeloma cells were dead, HAT complete media was replaced with HT complete media. The cells were fed four times more with HT then returned to normal media.

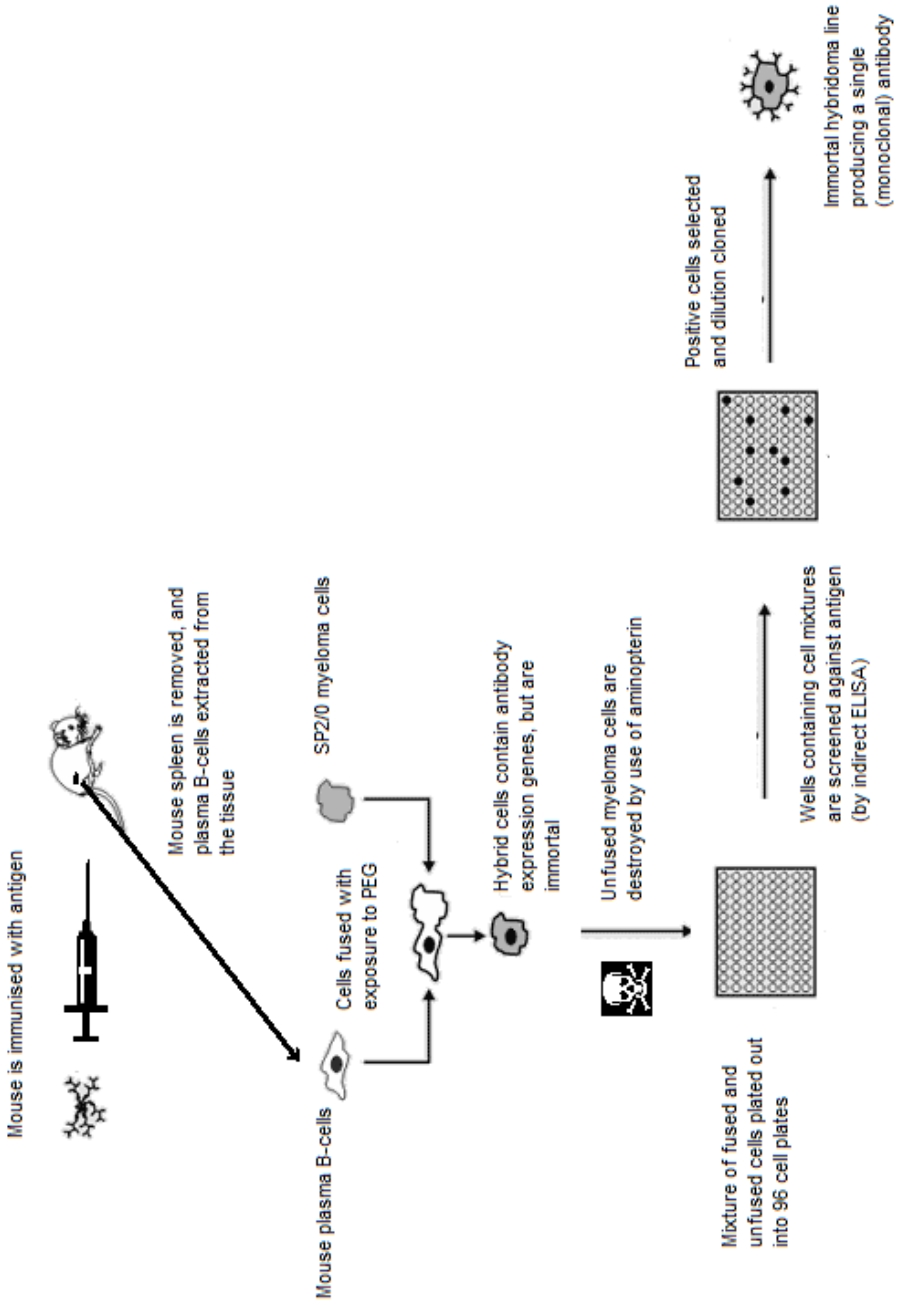



Figure 2.5: Simplified representation of the hybridoma fusion protocol.

### 2.6.4 Hybridoma screening

When the SP2/0 had visibly perished and hybridoma colonies had begun to grow to clusters of over 20 cells, the plates were screened for presence of anti-*Listeria* antibody.

Screening plates were prepared by dilution *Listeria* stocks to a concentration of $10^5$ cells/ml and combining all ten stains in an equal volume for a total concentration of $10^6$ cells/ml. The plates were then coated with this mixture at 100 µl/well, all wells and incubated for 1 hour at 37°C, washed, and dried.

The plates were then blocked and stabilised by the addition of 300 µl/well stabilisation buffer: 1% (w/v) casein and 0.25 M trehalose in 10 mM dPBS pH 7.4. This was incubated for 1 hour at 37 C, then aspirated rather than washed. The plates were then allowed to dry in a class 1 airflow hood then stored in a desiccator at 4°C until use. Preserved this way the plates would last almost indefinitely.

Upon feeding plates to be screened, the 100 µl of supernatant removed from the plate during the feeding process was not discarded but transferred to a sterile, lidless 96 well tissue culture plate. This was in turn transferred to the screening plate and incubated at 37°C for 1 hour at 37 C, washed, and dried.

Anti-mouse H+L HRP stock was diluted 1:2500 in blocking buffer: 3 % (w/v) casein in dPBS, and plated out at 100 µl/well, all wells. This was again incubated at 37°C for 1 hour, washed and dried.

250 µl of TMB stock was diluted in 25 ml of 0.1 M acetate-citrate buffer pH 5.5 supplemented with 5 µl 30% (v/v) $H_2O_2$ and plated out at 100 µl/well all wells. Colour was allowed to develop for 20 minutes at room temperature in the dark then the reaction stopped with the addition of 100 µl 1 M $H_2SO_4$ in all wells. The absorbance of the plate was then read at 450 nm. Positive wells were identified by an A450 absorbance value of greater than 0.100 up to 2 weeks after fusion, or 0.200 after this point.

When positive wells were more than 50% confluent the cells and supernatant were removed to 1 ml of complete RPMI in a 25cm tissue culture flask. Negative wells were screened several times to ensure that there was given sufficient time to produce antibody before being rejected.

When anti-*Listeria* positive wells were identified and moved into flasks, the flasks were named according to the plate number and well they were found in and cultured until they had around 5 ml and a reasonable number (10-30% confluent) of cells.

2 ml of supernatant was then removed for a second screening stage. ELISA plates were with $10^6$ cells/ml of each of the 10 *Listeria* strains in each column, as well as the two remaining columns as *E. coli* and *S. enteriditis* as negative controls. This was then incubated, washed, blocked, and run in all other ways as in the previous screening (Section 2.5.4).

Producing flasks were then cultured and split. Cell lines expressing anti-*Listeria* antibody of any kind were preserved by taking the contents of an entire 25 $cm^2$ flask near confluency (between $10^6$ and $2x10^6$ cells), suspending the cells by gentle shaking and pipetting the suspension into a 15 ml centrifuge tube. This was centrifuged at 600 RCF and the supernatant discarded. Care was taken to remove as much of the media from the pellet as possible. The pellet was then re-suspended in 1 ml freezing medium at 4°C and transferred to a cryovial. Vials were placed into a Mr. Frosty freezing container and placed in a -80°C freezer. After 3-7 days at -80°C, vials were transferred either to liquid phase nitrogen or -150°C ultra-low temperature freezer for indefinite cryopreservation storage.

### 2.6.5 Dilution cloning

With the producing cells secured, they could then be formed into monoclonal lines by dilution cloning. Cells from an antibody producing line were counted by haemocytometer and diluted to approximately 10 cells/ml in cloning media: complete RPMI with 20% (v/v) FBS. The increased FBS allowed better support for isolated hybridoma. The diluted cells were plated out four of the top row of wells on a 96 well

tissue culture plate, incubated for 30 minutes in 37°C and 5% $CO_2$, then inspected under the microscope. If there were too many cells in each well (ideally there should be one in around a third of them, more than one in a third and none in a third), then the cell suspension was again diluted accordingly.

The cell suspension was then plated out on the centre 64 wells of ten 96-well tissue plates and incubated for three days. They were then visually inspected by microscope; single cells by this point should have grown to small colonies of 4 to 18 cells which are easier to see. If any well contained more than one colony these were marked down.

After 7 to 9 days (depending on growth), the plates were screened for anti-*Listeria* production as in Section 2.5.4, although with the higher affinity *Listeria* strains as coating rather than an equal mixture. Clones which had grown from a single cell and produce antibody were then removed, cultured in 25 $cm^2$ flasks, cryopreserved and the cloning repeated one more with these cells.

### 2.6.6 Characterisation of an anti-*Listeria* monoclonal cell line

Tissue culture flasks containing F4-1F2-1C8 were left for 1 week without feeding to concentrate any excreted antibody. The supernatant was then harvested.

Microtitre plates were coated with $10^6$ relative cells / ml of *Listeria* standards in PBS, with one column for each of the 10 strains. Two attenuated non-*Listeria* were put in the remaining two columns at the same concentration: *K. zopfii* and *Br. laterosporus*. The plates were incubated (37°C for 1 hour), then washed and blocked with 3% (w/v) casein in PBS for a further hour at 37°C. After washing, F4-1F2-1C8 was then put in all wells at 200 µl/well and incubated. As a secondary, 100µl/well anti-mouse (H+L) HRP 1:2500 in 3% (w/v) casein/PBS was plated out in all wells and incubated, washed and dried. Uptima TMB was used as substrate.

To purify F4-1F2-1C8, 100 ml of supernatant was filtered through 0.22 µm PES filter (Millipore Stericup) and 8.7 g of solid potassium sulphate added (87 mg per ml of supernatant, resulting in 0.5 M potassium sulphate in the supernatant). This was allowed to dissolve with stirring in an end-over-end stirrer at room temperature for 1 hour.

1 ml of T-Gel resin (Pierce) was equilibriated with binding buffer (0.1 M phosphate, pH 7.0 + 0.5 M potassium sulphate). The supernatant was applied to the column at 0.5 ml/minute and the column washed with binding buffer until the 280 nm detector returned to baseline (approx. 200 ml buffer). The bound protein was then eluted with the addition of elution buffer, 0.1 M phosphate pH 7.0 + 0.135 M NaCl. It was then quantified at 280 nm.

To bind F4-1F2-1C8 to microtitre plates as a capture antibody, the purified antibody was diluted to 20 µ $ml^{-1}$ in elution buffer and plated at 100 µl/well. Incubation was 1 hour at 37°C, followed by a wash with PBS-T as in ELISA assays described in earlier sections, and blocking with 3% (w/v) casein in PBS for 1 hour, again followed by a wash.

## 2.7 Broth development for food testing

Developed before this study, Selective *Listeria* Broth (SLB) proved more than adequate for growth of *Listeria* as it supported rapid growth of all strains and in earlier tests had completely inhibited other ubiquitous test organisms such as *E. coli*. It had yet to be tested under real-world conditions with foods and the many micro-organisms they are host to: in particular gram positive bacteria and other members of the *Listeriacae*. Developed before this study, it is a relatively rich broth, and was intended more for rapidity of *Listeria* growth rather than for its selectivity, so it will inevitably require substantial modification.

Selective *Listeria* Broth ingredients are given in Materials and Methods. To support the rapid of growth of *Listeria* it contains tryptone, yeast hydrolysate and iron in the form of ammonium ferric citrate. All ingredients are autoclaved, accepting the potential reduction in activity from high temperature treatment as part of the overall formulation.

### 2.7.1 Selective *Listeria* broth evaluation with foodstuffs

Using the CLISA sandwich method, five representational foods were tested in SLB: infant formula milk powder, beef mince, red capsicum, chicken patê and haddock fillet. These were purchased from a high street supermarket in pre-packaged form. Just four representational *Listeria* strains were selected to simplify the test: clinically relevant *L. monocytogenes* serovars 4b, 1/2a and 1/2b; *L. innocua* as among the fastest-growing; *L. seeligeri* as among the slowest growing (*L. ivanovii* was slower but not as conclusively easy to identify using chromogenic agar). The official ISO method was run alongside the CLISA assay as a firm basis of comparison.

The chosen strains were cultured in SLB at 37°C. After centrifugation and washing, in a Class 2 cabinet to avoid contamination by other organisms, washed bacteria pellets were quantified by turbidity measurement at 540 nm to estimate cells/ml. On the basis of this, strains were then diluted to 3 CFU/ml and 30 CFU/ml in sterile saline the day before the food test. The 30 CFU/ml was counted by adding 100 µl to plate count agar (PCA) spread plates. The 3 CFU/ml stock was kept at 4°C overnight.

Each food was pre-assayed for total aerobic plate count (APC) when fresh using SimPlates according to their documentation to establish a bacterial load baseline.

Then 25 g of food was added to 225 ml of broth and stomached in a stomacher bag for 3 minutes by machine. Each *Listeria* combination in SLB was mirrored by the same combination in Half Fraser Broth. Each sample was then spiked with 3 CFU in 1 ml of saline with one un-spiked negative control. SLB + food samples were incubated at 37°C for 20 hours; Half Fraser Broth + food samples for 24 hours at 30°C.

Each SLB/food mix was then streaked onto PCA for counts and chromogenic agar (Rapid L mono and OCLA) and 3 ml taken and placed in a 5 ml polypropylene boiling tube, covered, and placed in a boiling water bath at 100°C for 20 minutes: these were then assayed by the sandwich CLISA method as in Section 2.4.3.

0.1 ml of each food and broth combination was put into 10 ml Full Fraser Broth and incubated 48 h at 37°C. This was also streaked onto the chromogenic agars for confirmation.

All bacterial growth on the chromogenic agars was then recorded and photographs taken. Isolated colonies of various morphologies were put on nutrient agar at 30°C for later investigation. Colonies suspected of being *Listeria* were tested by latex agglutination.

### 2.7.2 Identification of bacteria in food cultures

Various gram-positive organisms were encountered in the food test that did not prove to be *Listeria* yet were able to grow relatively well in SLB. Where possible single colonies were purified to homogeneous cultures by repeatedly sub-culturing on BHI agar, and when considered pure by morphological observation, these were stored on cryobeads and BHI agar slopes. Of particular interest were those bacteria present in large numbers in foods where the CLISA was either not successful in detecting *Listeria* or where background was high in samples not inoculated with *Listeria*.

Because no readily available test exists that can reliably identify a genus range as wide as, for example, “gram positive rods”, catalase test, oxidase test, gram stain, blood agar haemolysis, growth at 42°C, and growth in anaerobic conditions were all used to narrow down the possible genus of these contaminating organisms. Gram negative rods, where present, were identified by API 20E or API 20NE depending on cytochrome oxidase production (confirmed by Biomerieux Oxidase Reagent).

### 2.7.3 Investigation of potential antibiotic supplements to SLB

Having isolated bacteria from food tests that did not work as planned (either with high background on the negative controls or *Listeria* not detected in SLB but detected by the ISO method), these were then investigated to determine their inhibition of *Listeria* and their sensitivity to several antibiotics. *Enterococcus faecalis* was not isolated from

the foods during this test but since it was already available in the laboratory, a very common food contaminant, highly antibiotic resistant, and well documented to inhibit *Listeria*, it was investigated as well.

Testing was initially done by adding 12 g $l^{-1}$ agar-agar to *Listeria* Broth, autoclaving, cooling to 45°C in a water bath, adding any antibiotics to be tested, and plating out in Petri dishes. Several cephalosporins documented with low Minimum Inhibitory Concentrations (MICs) against *Listeria* were trialled: cephalothin, cefoxitin, cefsulodin and ceftazidime.

Acriflavine (a very common *Listeria* selective agent) and nitrofurantoin, a specialised anti-gram positive cocci antibiotic, were also investigated in combination with the cephalosporins. Acriflavine was also was tested despite only weak evidence of efficacy in agar trials.

Cultures of *Listeria* strains and isolated organisms were then streaked on the agar with visually inhibited growth after 24 hours at 37°C being taken as a qualitative result for further investigation.

When it became quickly apparent that this simplistic model did not mirror results in broth, the testing was focused on broth only and became more quantitative. Aliquots of 50 ml Selective *Listeria* Broth were inoculated with approximately 1000 CFU of *Listeria* strains with and without with a touch colony of competing organism (approximately $10^5$ CFU). The competing organisms used were *E. faecalis*, an unidentified haemolytic *Streptococcus* isolated in the food testing, and two coryneforms also isolated, one of which was suspected to be a species of *Kurthia*. After a 20 hour culture cycle at 37°C, 3 ml of broth was taken, immersed in boiling water at 100°C for 20 minutes, and assayed by CLISA.

### 2.7.4 Investigation of ceftazidime as a selective agent

Having settled on a number of different broth recipes that seemed to be non-inhibitory to *Listeria* but displaying increased sensitivity, the next logical step was to repeat testing on foods that failed to assay in CLISA with the original SLB.

Modifed Selective *Listeria* Broth (mSLB) was made by adding 12 mg $l^{-1}$ nitrofurantoin and 1.5 mg $l^{-1}$ acriflavine to 1 L SLB base and autoclaving for 15 minutes at 121°C. The broth was cooled to 50°C before 2 mg $l^{-1}$ ceftazidime was added.

See Section 2.6.1 for the protocol but note that in this case the ISO method was not run alongside. Foods tested were the foods that failed to assay properly by CLISA before: beef mince and cod fillet. Greek yoghurt was also tested.

### 2.7.5 *Listeria* growth quantification in SLB and mSLB

Growth rate of all of the selected *Listeria* species and serotypes was investigated to ensure that with addition of selective agents they were all able to grow at a rate equal to or close to that of the original SLB.

Screening plates were prepared by coating white 8 well microtitre strips with 100 µl of KPL BacTrace anti-*Listeria* at 2 mg $ml^{-1}$ in 0.1 M carbonate buffer pH 8.5. These were incubated at 37°C for 1 hour then washed and dried. They were then blocked with 1% (v/v) casein and 0.25 M trehalose in PBS for 1 hour at 37°C, washed, dried and stored at 4°C in a dessicator in the dark until needed.

*Listeria* was cultured by adding a loop from a slope to 40 ml of SLB and leaving overnight at 37°C. The *Listeria* was pelleted and washed three times by centrifugation and PBS and quantified by turbidity (see Section 2.6.1). The pellet was then diluted to what was assumed to be 100 CFU/ml and 1 ml used to inoculate 40 ml of SLB and mSLB. Using a sterile pipette tip, 100 µl was spread on BHI agar plates for an authoritative CFU count. Both plates and broths were cultured overnight at 37°C. At the 16 hour incubation mark, and at every 1 hour interval afterward up to 24 hours, 1

ml samples were taken from either broth and immediately frozen at -20°C. When all samples were collected they were then placed in a block heater at 100°C for 20 minutes and assayed by sandwich CLISA as in section 2.4.3.

### 2.7.6 Modification of broth for infant formula milk

To explore broth components concerned with dealing with *Bacillae* in infant formula milk, chromogenic agars combined with visual inspection was used.

Cefotetan viability as an additive was determined by visual inspection of round green *Listeria* colonies versus irregularly shaped bacillus colonies.

Culture was as before in sections 2.6.3 and 2.6.4, but with a smaller scale. 2.5 g of food was added to 22.5 ml of broth in a 50 ml sterile polypropylene centrifuge tube and incubated at 37°C for 20 hours. It was then streaked onto OCLA and Rapid L mono agar and incubated at 37°C for 24 hours and visually inspected.

# Chapter 3: Results

## 3.1 Establishment of live *Listeria* stocks

### 3.1.1 Validation of *Listeria* stocks

The tables show the results of the various tests on the *Listeria* stocks subsequently stored on cryobeads as permanent stocks. The Health Protection Agency (HPA) reported the results of their identification and serotyping of samples sent to them as in Table 3.1.

There are some differences between the results reported by HPA and the results in this study. It is possible, though very unlikely, that the slopes of the bacteria that differed from expected results were contaminated when sent to the HPA. This cannot be confirmed as only one slope of each strain was sent.

In the cases of (A) and (C), both *L. monocytogenes*, the serotype differs only by designation terminology. There are two serotyping designations for *Listeria*: the original Paterson, and the Seeliger-Doenker-Voet. Serotype 1 Paterson is identical to 1/2a Seeliger-Doenker-Voet, while 4 Paterson is also designated 4a Seeliger-Doenker-Voet. Therefore both serotypes are correct. It is not clear why HPA chose to mix designations and no explanation was given, but it is possible that the serotyping process they used simply reported these designations.

HPA also did not completely identify (F) *L. ivanovii*. The report on the sample commented: "This isolate could not be fully identified to the species level by 16s sequencing having 99% homology to both *L. ivanovii* and *L. seeligeri*. Phenotypic identification by API gave a profile that was 82.2% similarity with *L. seeligeri* and 17.6% to *L. ivanovii*."

Although (G) *L. seeligeri* was correctly identified, it was also tagged with a HPA report comment: "Our initial testing by Real time PCR confirmed the strain as *Listeria* spp. Further testing by 16s sequencing has confirmed the strain as *L. seeligeri*."

Results of identification carried out directly by API *Listeria* and chromogenic agar (OCLA, Blood Agar) are shown in Table 3.2. Unlike HPA's API test, there was no difficulty positively identifying *L. ivanovii* (at 99.9%) although the certainty of the *L. seeligeri* API was weakest (94.2%, below the 95% confidence level). This is warned of in the API *Listeria* documentation as a limitation of the test rather than being a possible contamination. On the basis of our own tests and the HPA results, all strains were accepted as being correct and suitable for all further work.

Table 3.1: HPA report on identity of supplied *Listeria* spp.

| Ref | Aquired Species (LMG) | Identified Species (HPA) | Identified Species (Agar/API) | Purchased Serovar (LMG) | Identified Serovar (HPA) |
|---|---|---|---|---|---|
| **A** | *L. monocytogenes* | *L. monocytogenes* | *L. monocytogenes* | **1** | **1/2a** |
| **B** | *L. innocua* | *L. innocua* | *L. innocua* | 6a | N/A |
| **C** | *L. monocytogenes* | *L. monocytogenes* | *L. monocytogenes* | **4b** | **4** |
| **D** | *L. welshimeri* | *L. welshimeri* | *L. welshimeri* | 6b | N/A |
| **E** | *L. monocytogenes* | *L. monocytogenes* | *L. monocytogenes* | 4a | **Non typable** |
| **F** | *L. ivanovii* | ***Listeria* spp. *(seeligeri)*** | *L. ivanovii* | 5 | N/A |
| **G** | *L. seeligeri* | *L. seeligeri* | *L. seeligeri / ivanovii* | 1/2b | N/A |
| **H** | *L. grayi* | *L. grayi* | *L. grayi* | | N/A |
| **I** | *L. monocytogenes* | *L. monocytogenes* | *L. monocytogenes* | 1/2b | 1/2b |
| **J** | *L. monocytogenes* | *L. monocytogenes* | *L. monocytogenes* | 1/2a | 1/2a |

Table 3.1: Each of the *Listeria* species along with its specification as published by LMG and its species and serotype as identified by the Health Protection Agency. It is likely that any disparities are due to differing test protocols and not due to contamination of *Listeria* stocks.

**Table 3.2:** Results of API identification of Listeria stocks

| Ref | Listeria Species/Serotype | OCLA Result | Expected β-haemolysis | Observed β-haemolysis | API Result and % ID reliability |
|---|---|---|---|---|---|
| **A** | *L. monocytogenes* 1 | *L. monocytogenes* | + | + | *L. monocytogenes* (99.9%) |
| **B** | *L. innocua* | Other *Listeria* | - | - | *L. innocua* (99.6%) |
| **C** | *L. monocytogenes* 4b | *L. monocytogenes* | + | + | *L. monocytogenes* (98.5%) |
| **D** | *L. welshimeri* | Other *Listeria* | - | - | *L. welshimeri* (99.9%) |
| **E** | *L. monocytogenes* 4a | *L. monocytogenes* | + | + | *L. monocytogenes* (98.6%) |
| **F** | *L. ivanovii* | Other *Listeria* | + | + | *L. ivanovii* (99.9%) |
| **G** | *L. seeligeri* | Other *Listeria* | + | + | *L. seeligeri* (94.2%) |
| **H** | *L. grayi* | Other *Listeria* | - | - | *L. grayi* (98.8%) |
| **I** | *L. monocytogenes* 1/2b | *L. monocytogenes* | + | + | *L. monocytogenes* (98.6%) |
| **J** | *L. monocytogenes* 1/2a | *L. monocytogenes* | + | + | *L. monocytogenes* (98.6%) |

Table 3.2: API *Listeria* tests on the *Listeria* species live stocks used in this study. They were correctly identified with high certainty of reliability. OCLA results can only determine if a *Listeria* species is *L. monocytogenes*.

## 3.2 Preparation of *Listeria* antigen

### 3.2.1 Growth of *Listeria*

All *Listeria* tested positive when plated on OCLA without selective component added, and displayed no mixed colonies. This was taken as indicative as a pure culture. Different strains of *Listeria* produced different growth rates and colony size, but colonies were of a single morphology: round, slightly shiny and convex. They were strongly coloured green, identifying them as being able to reduce glucose. *Listeria monocytogenes* colonies had a cloudy aura around them due to lecithinase activity.

### 3.2.1 Enumeration of *Listeria*

In order to produce stocks with a standardised quantity of *Listeria*, it was necessary to enumerate *Listeria* in a reproducible way. Enumeration was achieved by turbidity measurement of cells as described in 2.3.2. The dilution factor was used to derive the concentrations in cells per ml shown in Table 3.3. This concentration includes dead cells as well as living ones.

Table 3.3: Boiled standards as enumerated by turbidity at 540 nm

| Ref | Spcies | Serovar | ATCC/LMG | Volume Saline (µl) | Volume OriginalStock (µl) | A540 | Estimated pre-boiling cellular conc. of Stock (cells/ml) |
|---|---|---|---|---|---|---|---|
| **A** | *L. monocytogenes* | 1 | ATCC19111 | 600 | 30 | 0.851 | $2.1x10^8$ |
| **B** | *L. innocua* | | ATCC33090 | 600 | 30 | 0.888 | $2.1x10^8$ |
| **C** | *L. monocytogenes* | 4b | ATCC13932 | 650 | 30 | 0.924 | $2.27x10^8$ |
| **D** | *L. welshimeri* | | ATCC3589 | 600 | 30 | 0.895 | $2.1x10^8$ |
| **E** | *L. monocytogenes* | 4a | ATCC19114 | 600 | 45 | 0.898 | $1.43x10^8$ |
| **F** | *L. ivanovii* | | ATCC19119 | 650 | 40 | 0.88 | $1.73x10^8$ |
| **G** | *L. seeligeri* | | ATCC35967 | 600 | 30 | 0.882 | $2.1x10^8$ |
| **H** | *L. grayi* | | ATCC19120 | 600 | 32 | 0.882 | $2.1x10^8$ |
| **I** | *L. monocytogenes* | 1/2a | LMG16780 | 600 | 30 | 0.918 | $2.1x10^8$ |
| **J** | *L. monocytogenes* | 1/2b | LMG23190 | 600 | 30 | 0.906 | $2.1x10^8$ |

Table 3.3: Volumes of boiled *Listeria* stocks and dilutions required to dilute them to a turbidity of approximately 0.900 absorbance units (540 nm). The concentration of *Listeria* cells per ml was derived using the formula in section 2.2.2.

## 3.3 Development of sandwich CLISA

### 3.3.1 Initial evaluation of KPL antibody by indirect ELISA

According to KPL, BacTrace Anti-*Listeria* High-Sensitivity may also show occasional low level cross-reactivity to *Staphylococcus* and *Streptococcus* species under certain test conditions. This was not initially investigated as this would have been relevant only if these bacteria were capable of growing in SLB. Although it was certified to detect all *Listeria* spp. it did not state whether it would detect only heat labile surface antigens or whether it would also be specific to boiled bacteria. It also did not specify whether the bacteria could be cultured at 30°C or 37°C.

KPL BacTrace was evaluated for its response to the selected *Listeria* strains by indirect ELISA, at a series of dilutions. The dilution concentration refers to an approximated equivalent number of *Listeria* cells per ml, and was diluted from the *Listeria* stocks prepared in section 3.2.2. Figure 3.4 shows the ELISA response of the KPL antibody toward the ten strains of *Listeria*.

Response was excellent, with all strains clearly being detected at equivalent $10^4$ cells/ml by the detection antibody and secondary HRP-labelled antibody. Most importantly, KPL BacTrace works with bacteria grown at 37°C and boiled and also with all strains and species. Many commercially available anti-*Listeria* antibodies tested prior to this study did not have this specificity.

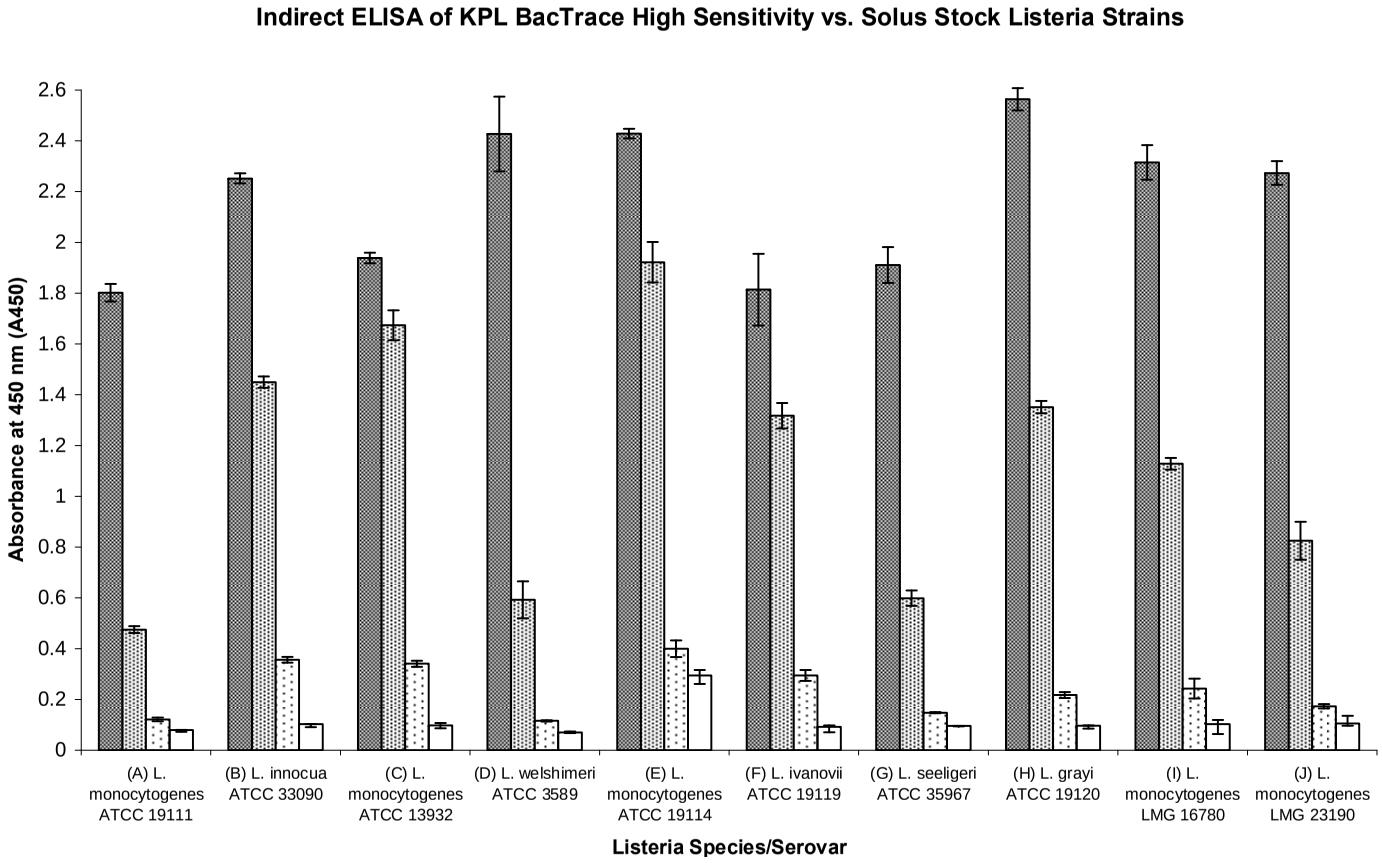



Figure 3.4: An indirect ELISA assay conducted various species and cultures of *Listeria* (A-J). From left to right, each of the columns represents $10^6$, $10^5$, $10^4$ and $10^3$ *Listeria* cells/ml.

### 3.3.1 Acridinium labelling of KPL antibody

The fractions collected from desalting acridinium labelled KPL BacTrace gave light output concentrated in the first few fractions (Figure 3.5). Fractions 3, 4 and 5 were pooled as the majority of labelled antibody is present in these fractions. The other fractions may also have contained antibody but these were discarded as they may also have contained appreciable quantities of unlabelled acridinium, i.e. acridinium-lysine. The acidic pH (pH 6.3) stabilises the acridinium label and the BSA acts as a protein carrier to prevent losses of antibody to the column and to plastic surfaces. Sodium azide prevents bacterial spoilage as the conjugate was not stored frozen.

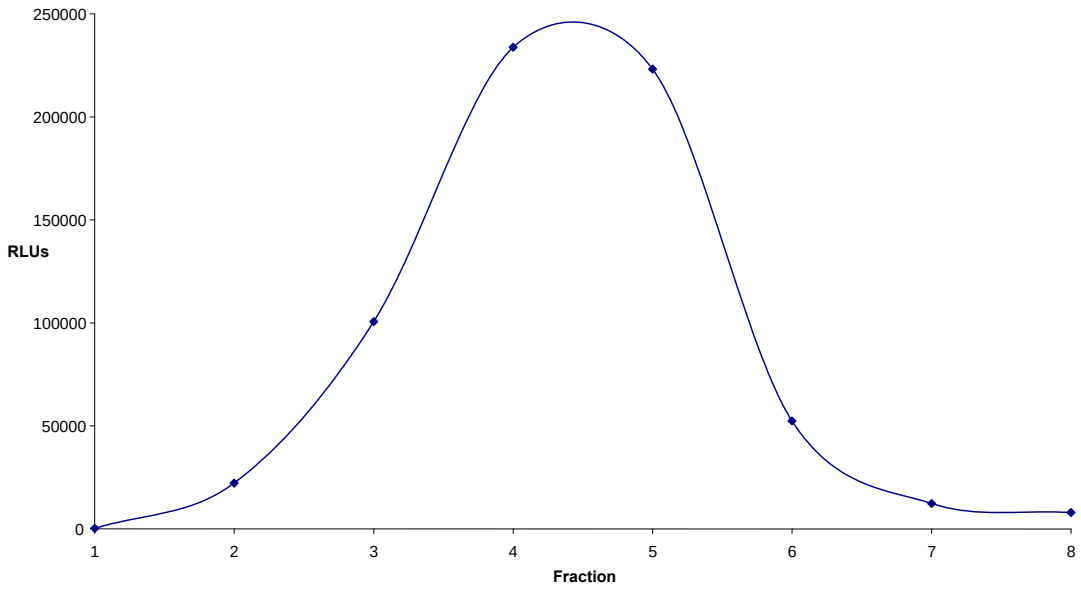



Figure 3.5: Acridinium ester light output by fraction. Points are joined only for visual effect.

### 3.3.3 Development of sandwich CLISA

The intention was to develop an assay that will detect *Listeria* in the presence of food matrices. Since in a typical direct or indirect immunoassay surface binding space in the titre wells would have to compete with food materials, a sandwich immunoassay format was chosen instead. In this format, the microtitre plate was first coated with anti-*Listeria* antibody to "capture" any *Listeria* antigens in the food. The food is then washed away before the detection antibody is used, preventing matrix effects. During assay development, broth containing *Listeria* inoculum was used without addition of food.

The sandwich CLISA employing KPL BacTrace both as detection and a capture antibody was successful in detecting all strains of *Listeria* tested. This confirmed that not only is a sandwich assay using only this antibody viable, but also that labelling the antibody with acridinium without damaging its specificity is successful.

CLISA light output is shown in Figure 3.7. *Listeria welshimeri* and *L. monocytogenes* serotype 4b produce the highest light output (and hence greatest antibody binding). It should be noted that CLISA response is linear, while ELISA response is logarithmic (light absorbance of a chromogenic substrate).

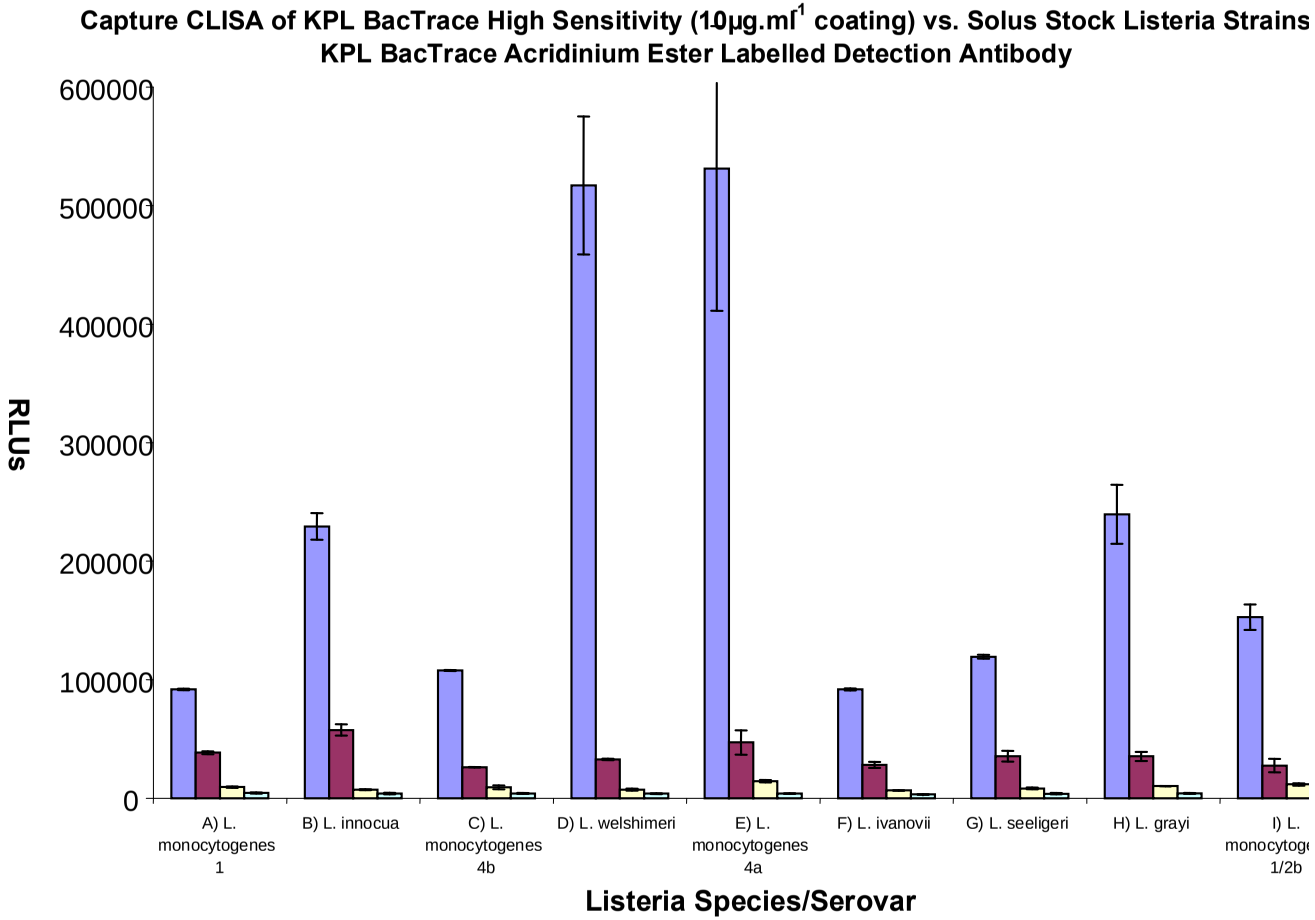



Figure 3.6: CLISA of *Listeria* using KPL BacTrace as both capture and detection Ab. From left to right, each of the columns represents $10^6$, $10^5$, $10^4$ and $10^3$ cells/ml of *Listeria.*

## 3.4 Raising of polyclonal antibody against *Listeria*

### 3.4.1 Pre-screening of sheep for endogenous anti-*Listeria* antibody titre

The sheep used were raised by SNBTS and were kept in open fields isolated from other animals but kept with other sheep used for similar purposes. They were not kept in sterile laboratory conditions therefore widely pre-existing immunity to common species of bacteria was expected. This first had to be investigated by screening serum from potential immunisation candidates against the ten validated *Listeria* strains, and also common commensal organisms such as *E. coli* and *S. enteriditis*. Rather than be related to *Listeria* or provide any potential problem as to cross-reactivity under assay conditions, *E. coli* and *S. enteritidis* were selected as markers to exclude sheep that had high existing antibody titre as unsuitable for immunisation.

Conversely, those sheep with an already existing high titre of anti-*Listeria* antibodies were provisionally decided more desirable for a starting point. The rationale was that sheep with high existing levels of anti-*Listeria* antibodies might be the best subjects for immunisation. The ELISA response of six sheep sera is displayed in Figure 3.8. Only sheep 111 did not have any significant anti-*Listeria* pAb titre.

In conclusion, sheep 107, 108 and 111 were chosen to be immunised. Sheep 107 and 108 had good existing titre toward *Listeria* that would possibly make immunisation more effective. Sheep 111 had very low titre towards overall and was therefore a good candidate for a “blank canvas” immunisation should cross-reactivity due existing pre-immunity be problematic later. Sheep 109 and 112 were rejected due to high existing titre towards *E. coli* and *S. enteriditis*, demonstrating the possibility of broad exposure to bacterial infection and therefore high cross-reactivity.

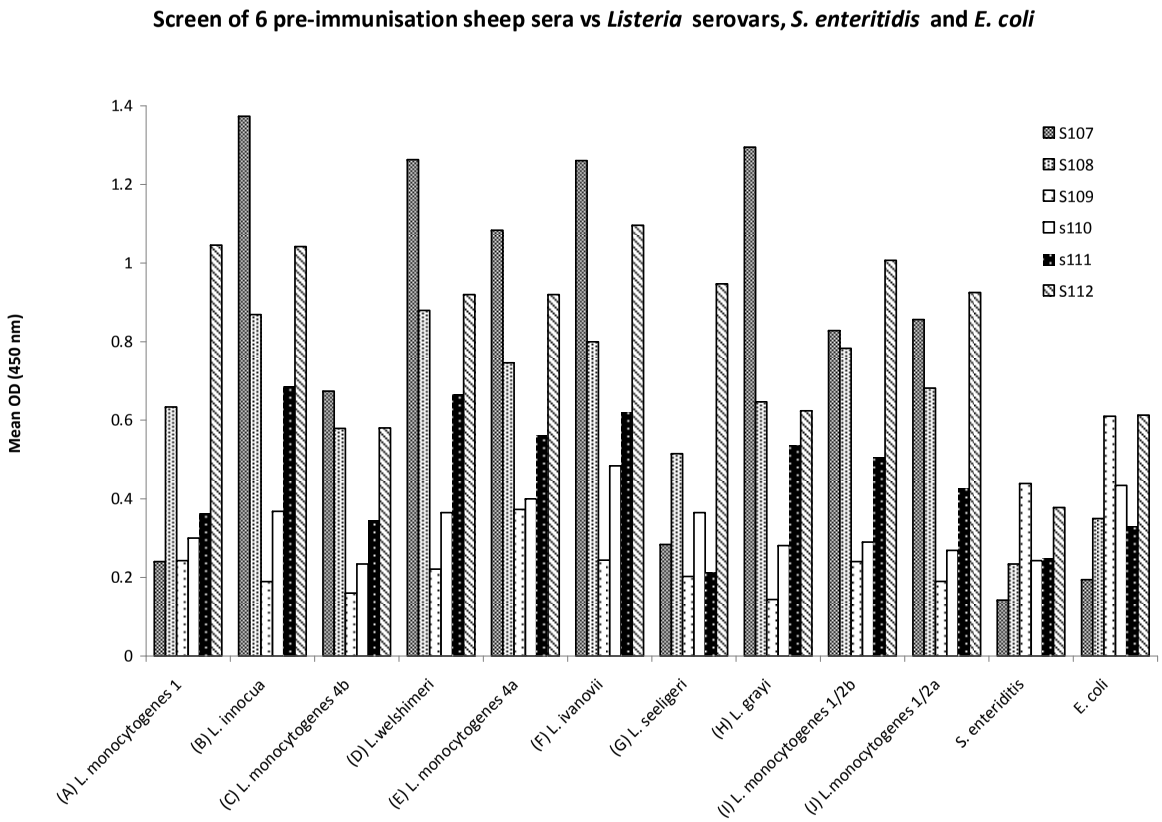



Figure 3.7: ELISA screening of pre-immune sheep sera against all *Listeria*. All sheep tested have some level of pre-existing anti-*Listeria* polyclonal antibody. This is strongest in sheep 107 and weakest in sheep 111.

### 3.4.2 Immunisation of sheep with attenuated *Listeria*

Three sheep were immunised with heat attenuated *Listeria* in order to raise polyclonal antibody: SNBTS sheep 107, 108 and 111, which were reassigned the letters A, B and C respectively. The individual sheep were immunised with a different mix of *Listeria* strains.

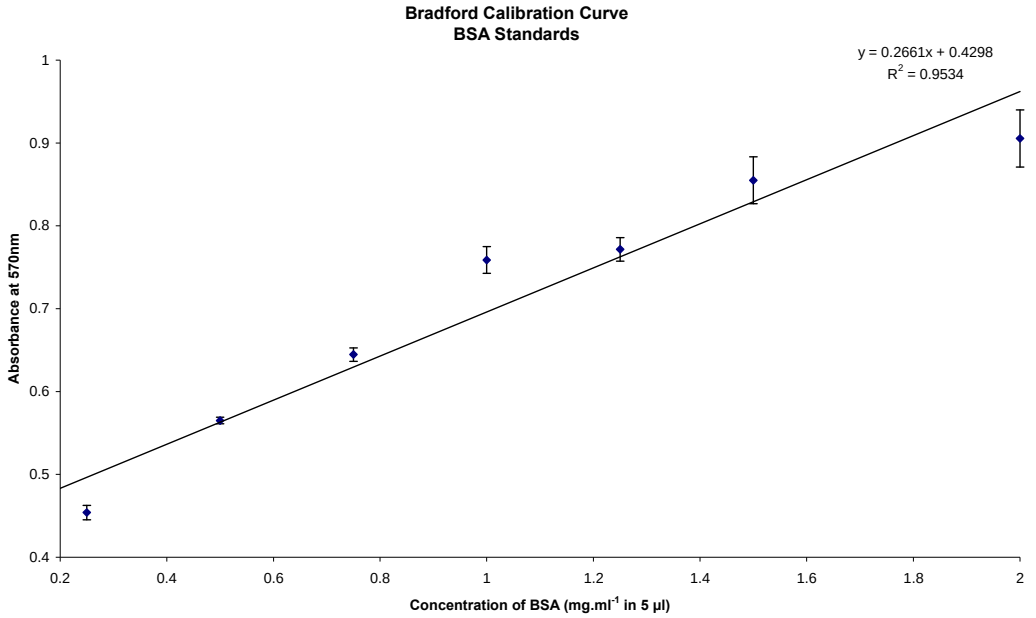
Sheep A, which had the highest pre-immune anti-*Listeria* titres, was selected to receive the four heat-attenuated strains that had performed best in ELISA and CLISA. While not necessarily anything more than a attribute of KPL BacTrace with reference to screening performance, there existed the possibility that these strains had some physical difference from the others that resulted in less immunogenicity. Sheep C, which had the lowest pre-immune titres, was given the three most poorly performing strains (*L. monocytogenes* serovars 1 and 4b, *L. seeligeri*). Sheep B was given the remaining three strains. The immunisation groupings and their justification are summarised in Table 3.8.

Table 3.8: Immunisation pools (sheep)

| Strain | Serovar | ATCC/LMG | | CLISA Response | ELISA Response | Sheep immunised |
|---|---|---|---|---|---|---|
| *A) L. monocytogenes* | 1 | ATCC19111 | | + | + | Sheep C |
| *B) L. innocua* | | ATCC33090 | | +++ | +++ | Sheep B |
| *C) L. monocytogenes* | 4b | ATCC13932 | | ++ | ++ | Sheep C |
| *D) L. welshimeri* | | ATCC3589 | | ++++ | ++++ | Sheep A |
| *E) L. monocytogenes* | 4a | ATCC19114 | | ++++ | ++++ | Sheep A |
| *F) L. ivanovii* | | ATCC19119 | | + | + | Sheep C |
| *G) L. seeligeri* | | ATCC35967 | | ++ | + | Sheep A |
| *H) L. grayi* | | ATCC19120 | | +++ | ++++ | Sheep A |
| *I) L. monocytogenes* | 1/2a | LMG16780 | | ++ | +++ | Sheep B |
| *J) L. monocytogenes* | 1/2b | LMG23190 | | ++ | ++ | Sheep B |

In order to determine the correct amount of bacterial antigen to be injected into the sheep for immunisation it was necessary to estimate the protein concentrations within the antigen samples. The Bradford calibration curve of Bovine Serum Albumin (BSA) is shown in Figure 3.9.

Figure 3.9: Bradford protein quantification standard curve




The linear regression formula derived (y = 0.2661x + 0.4298) was then used to derive y for x (concentration): x = (y – 0.4298) / 0.2661. The neat stocks were too concentrated to fall within the range of standards, so the 1:4 dilution absorbance values were charted and extrapolated.

Table 3.10: Derived protein concentrations of *Listeria* stocks

**Derived Concentrations**

| Stock | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1:4 Dilution (mg ml$^{-1}$) | 1.67 | 2.41 | 2.31 | 2.01 | 2.32 | 1.97 | 1.41 | 1.92 | 1.92 | 2.03 |
| Original Conc. | 6.67 | 9.65 | 9.24 | 8.05 | 9.26 | 7.87 | 5.65 | 7.66 | 7.66 | 8.13 |

(Please see Table 3.9 for *Listeria* stock letter designations.)

For sheep B and C, boiled bacterial stocks were diluted to protein concentrations of 160 µg ml$^{-1}$. 417 µl of each stock was then pooled with the other assigned stocks for these sheep to give a final volume of 1.25 ml for immunisation. For sheep A, stocks were diluted to 160 µg ml$^{-1}$ and 312 µl of each pooled together for each 1.25 ml immunisation. Figure 3.11 shows the result of ELISA screening of the sera against *Listeria* on the after the first and second immunisations.

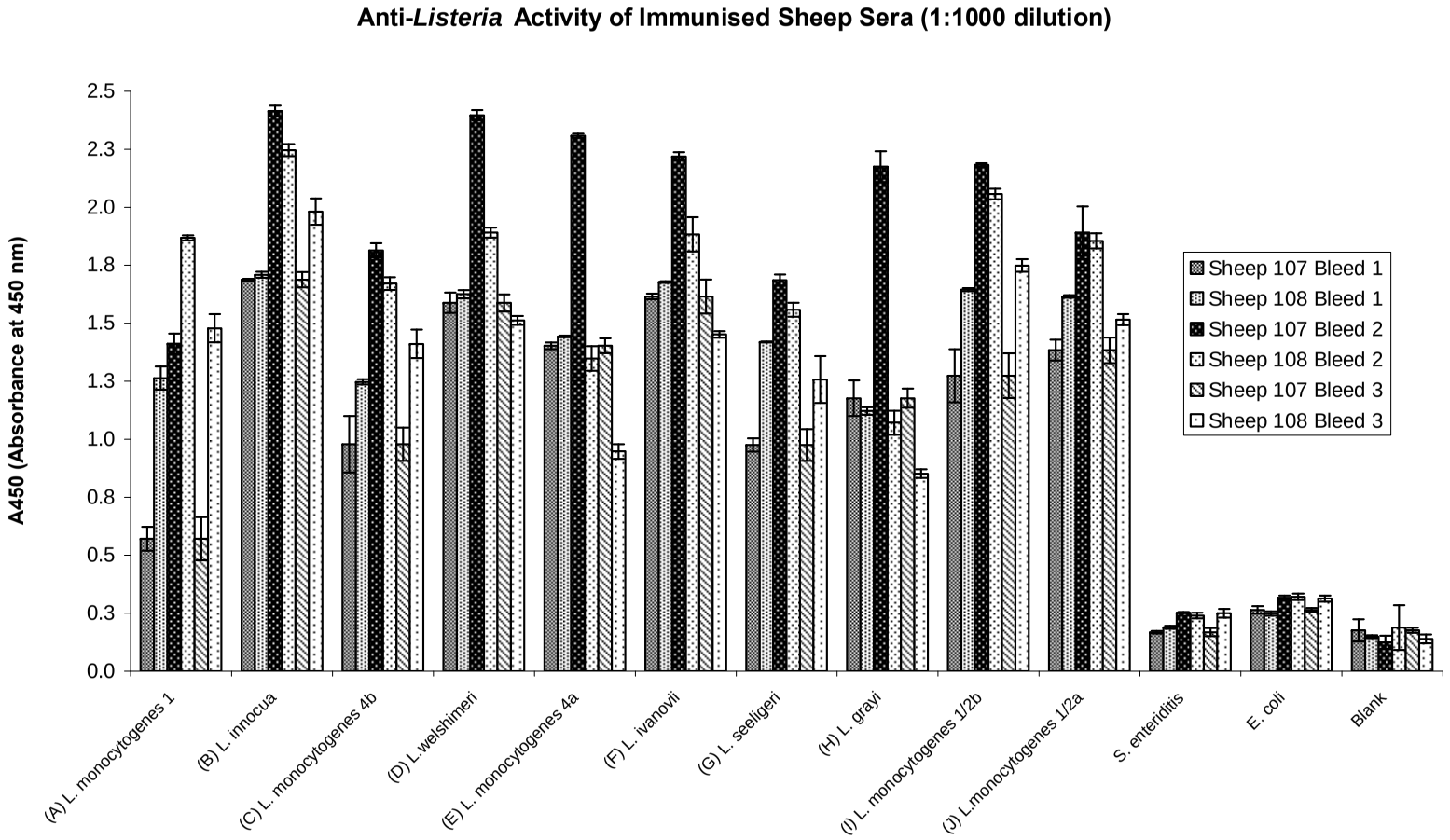



Figure 3.11: ELISA displays an increase in serum anti-*Listeria* antibody titre between the first and second sheep immunisations. Error bars are ±1 standard deviation.

### 3.4.3 Immunoaffinity purification of sheep polyclonals

Sheep serum was passed through sepharose 4b resin with *Listeria* cell components covalently bound to its surface area. When an elution buffer of pH 2.7 (0.1 M glycine HCl) was passaged, the sheep antibody bound to this antigen was eluted according to a peak in the 280 nm reading.

After dialysis, the protein in the pooled peak was quantified at 280 nm and accounted for 450 μg of total antibody from 10 ml of serum.

### 3.4.4 Capture CLISA with immunoaffinity purified sheep pAb

The possibility of using immunoaffinity purified sheep pAb as the detection component of a capture assay was investigated using a capture CLISA. KPL BacTrace was chosen to be the capture antibody component, as it was proven to work when employed in this way Figure 3.12 shows the CLISA response.

The sheep pAb appear to be effective in detecting all *Listeria*, although the RLUs are substantially lower than those of the CLISA using acridinium-labelled BacTrace as a detection antibody.

Unlike KPL BacTrace however, the non-*Listeria* show no response above the negative control (with the possible exception of *E. faecalis*). Importantly, *Kurthia* and the *Bacillus* from formula milk are not detected.

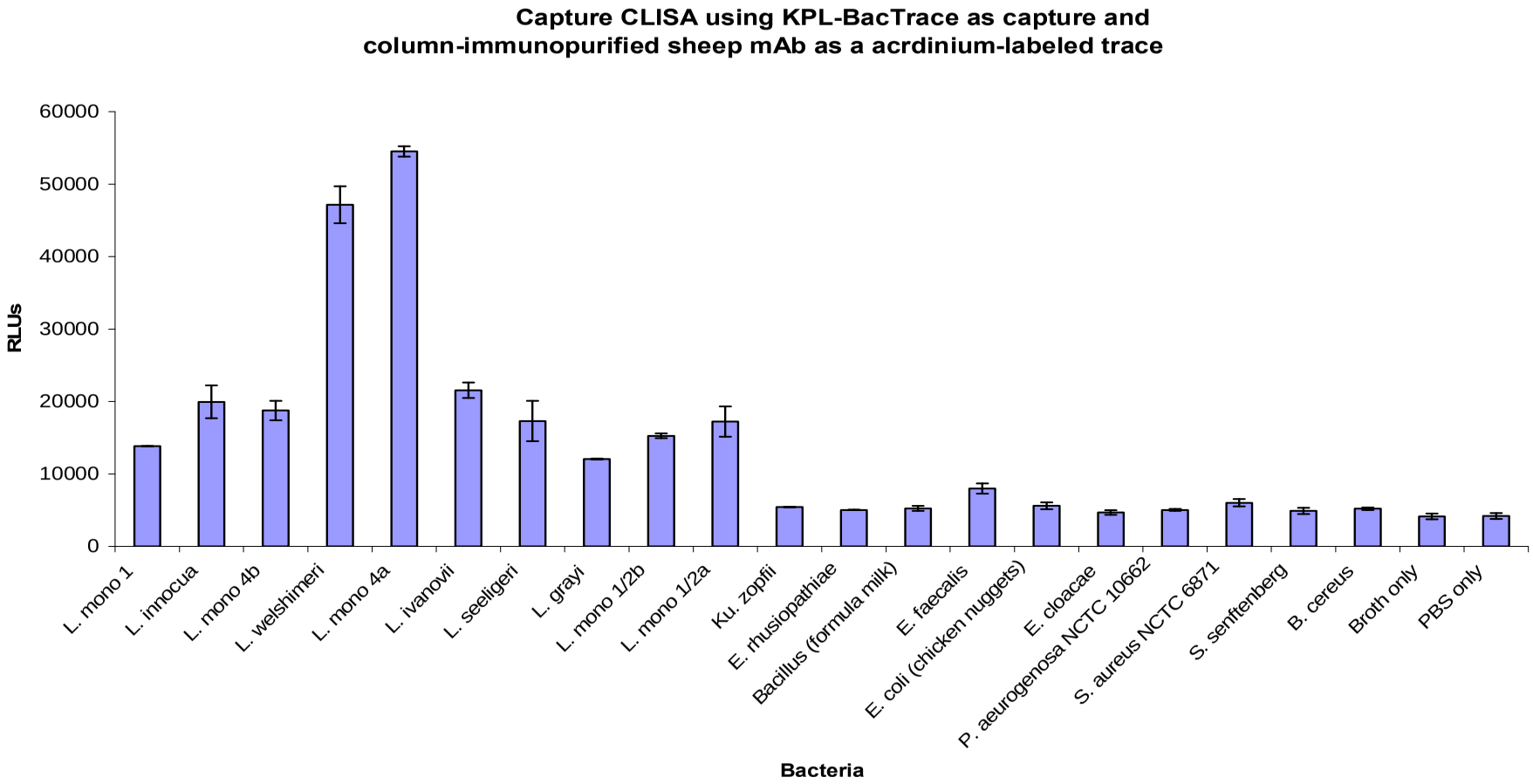



Figure 3.12: *Listeria* sandwich assay using KPL BacTrace as a capture Ab and immunopurified sheep pAb labelled with acridinium ester as a detection antibody. While the LRUs are substantially lower than KPL in both roles (Section 3.3.3), cross-reactivity toward non-*Listeria* bacteria is not evident. Error bars are ±1 standard deviation.

### 3.4.5 Assessment of an existing *Listeria* ELISA

As a basis of comparison with KPL Bactrace and the sheep pAb, *Listeria* stocks grown at 30°C and 37°C in mSLB were run at $10^6$ relative cells/ml in mSLB diluent on a Bioline *Listeria* assay kit. Figure 3.14 shows the kit results at with the *Listeria* grown at 30°C, which the kit is certified to be able to detect. Figure 3.15 shows how the kit performs with stock *Listeria* grown at 37°C used throughout this study.

The Bioline kit appears not to be able to detect the tested strain of *L. seeligeri*, and its response to *L. grayi* is weaker but still easily distinguishable from background. According to its AFNOR accreditation, it should have been capable of detecting all *Listeria*. The standard has been validated as *L. seeligeri* and is detectable as *Listeria* by both KPL and sheep serum, so this failure is certainly not due to a contaminated *Listeria* stock.. It is possible that this strain of *L. seeligeri* was not that used in the accreditation trials, but according to its ATCC number it should be identical. This is most certainly a weakness in the kit.

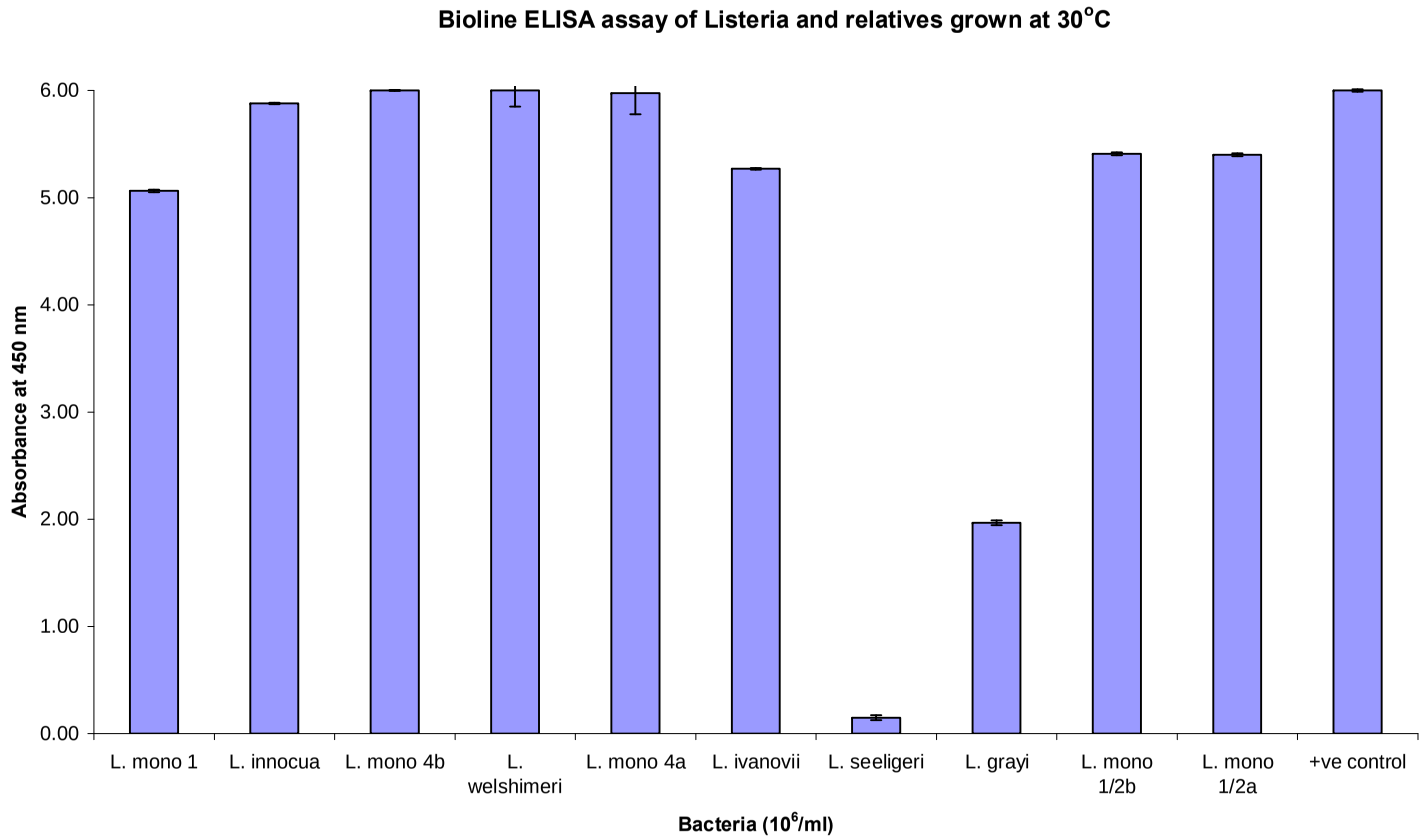



Figure 3.13: Bioline kit results with *Listeria* cultured at 30°C ($10^6$ cells/ml), as per kit instructions. The plate reader used measures absorbance up to 6.0, but anything above 3.0 can be taken as having saturated the assay. All strains are detected, with the exception of *L. seeligeri*. *L. grayii* detection is weaker than the others but still off scale. Error bars are ±1 standard deviation.

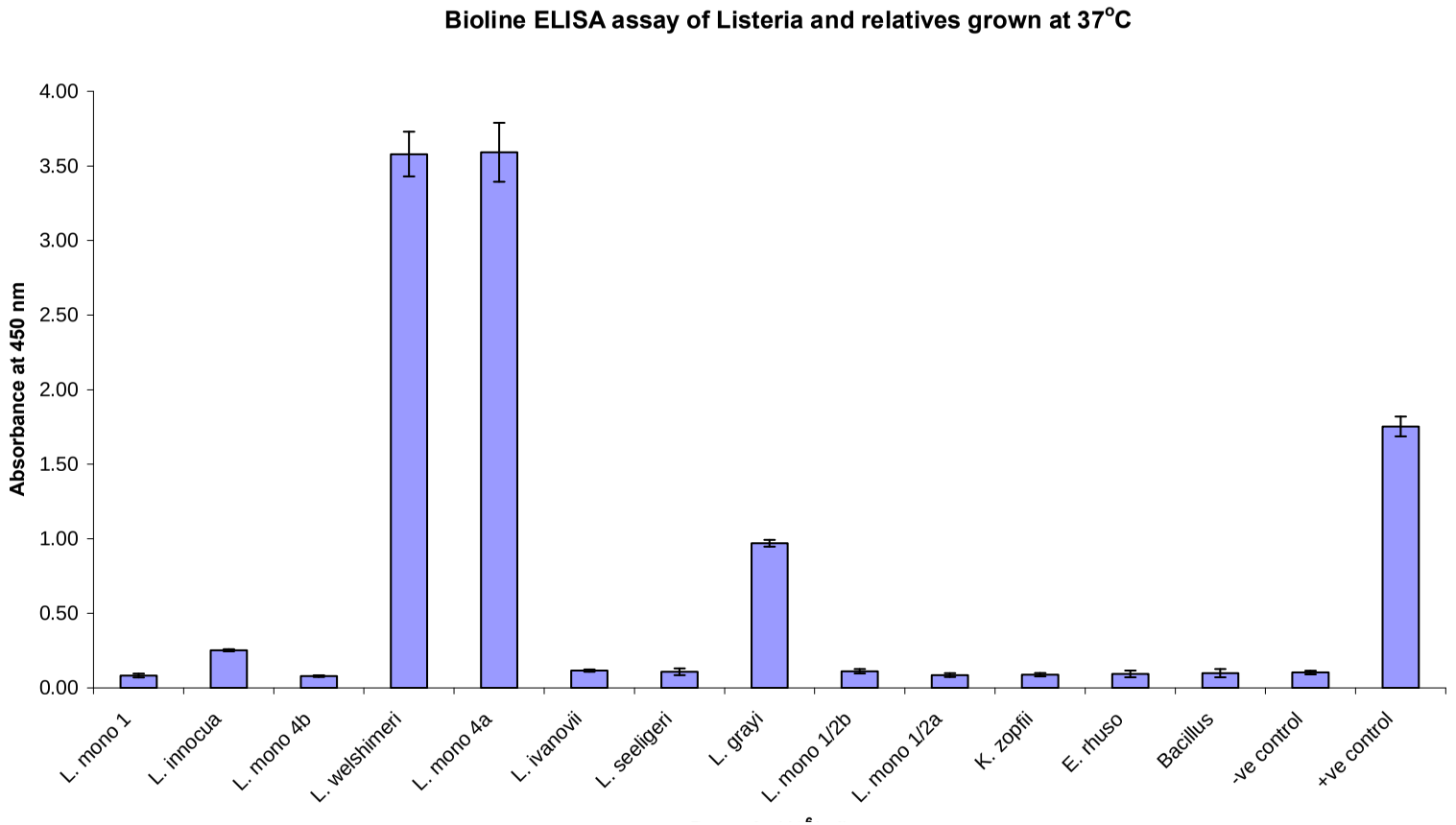



Figure 3.14: Bioline kit results with *Listeria* cultured at 37°C. The kit failed to detect many of the strains, as it is specified as anti-flagella and flagella should not be expressed at this growth temperature. It is notable that *L. monocytogenes* 4a, *L. welshimeri* and *L. grayi* still express flagellar antigen even when cultured at this temperature.

### 3.4.6 Capture CLISA using immunopurified sheep pAb only

A CLISA was run using anti-*Listeria* immunopurified sheep polyclonal antibody as both the coating (capture) and as a detection antibody. Only a positive control (*L. monocytogenes* serotype 4b) and a negative control (*K. zopfii*) were run, in order to assess background.

Table 3.15: Recorded RLUs vs pAb concentrations (against *L. mono* 4b)

| ***Acridinium labeled pAb Dilution factor*** | **Coating pAb concentration (µg ml$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **1** | **2** | **5** | **10** | **20** |
| ***1:40*** | 12036 | 45609 | 88427 | 105955 | 244547 | 397819 |
| ***1:60*** | 5976 | 23464 | 57134 | 62610 | 144476 | 232314 |
| ***1:80*** | 5652 | 33309 | 54581 | 57546 | 124449 | 152596 |
| ***1:100*** | 3935 | 28208 | 42740 | 42309 | 104794 | 103969 |

Table 3.15 shows the recorded RLUs for the combinations of coating and detection antibody against the positive control.

Table 3.16: Recorded RLUs vs pAb concentrations (against *K. zopfii*)

| ***Acridinium labeled pAb Dilution factor*** | **Coating pAb concentration (µg ml$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **1** | **2** | **5** | **10** | **20** |
| ***1:40*** | 9770 | 21432 | 50958 | 54081 | 135674 | 217866 |
| ***1:60*** | 5329 | 16553 | 32330 | 36286 | 78038 | 114943 |
| ***1:80*** | 4345 | 20150 | 30601 | 32236 | 64755 | 77183 |
| ***1:100*** | 3021 | 13912 | 22260 | 27326 | 46398 | 55333 |

Table 3.16 shows the recorded RLUs for the negative control. If the combination of antibodies had worked optimally, these should all be close to or identical to the non-coated strips (0 µg ml$^{-1}$).

Background on the negative control is extremely high, exceeding half of the positive (*Listeria*) response. Referring to Section 3.4.4, it is important to reiterate that this pAb does not bind to *K. zopfii* with any level of activity of the scale observed here.

This background increases as the level of sheep antibody increases, and its proportion to the anti-*Listeria* signal seems to remain approximately constant. This shows that the background must be due to sheep pAb binding to sheep pAb and not to *K. zopfii*. If it were binding to the negative control bacteria, signal would be expected to rise only marginally as pAb concentrations rose as the limited amount of antigen would soon become saturated.

## 3.5 Raising of monoclonal cell lines

### 3.5.1 Immunisation of mice

*Listeria* stocks prepared in Section 3.2.2 were used to make mouse immunogens after heat attenuation and prior to any use in ELISA or CLISA assays as standards so they were sterile when injected.

The following table (Table 3.17) shows the amount of each stock used per 1 ml of immunogen for both the $10^7$/ml group and the $3x10^7$/ml group of mice. Once each stock had been added, the remainder was brought up to 1 ml with sterile saline. Several aliquots, one per group per injection, were made.

Table 3.17: *Listeria* strains used for immunisation

| Ref | Species | Serovar | ATCC/LMG | Conc. Of Stock (cells/ml) | µl Stock used for 1 ml of $10^7$/ml | µl Stock used 1 ml of $3x10^7$/ml |
|---|---|---|---|---|---|---|
| A | *L. monocytogenes* | 1 | ATCC19111 | $2.1x10^8$ | 4.8 | 14.3 |
| B | *L. innocua* | | ATCC33090 | $2.1x10^8$ | 4.8 | 14.3 |
| C | *L. monocytogenes* | 4b | ATCC13932 | $2.27x10^8$ | 4.4 | 13.2 |
| D | *L. welshimeri* | | ATCC3589 | $2.1x10^8$ | 4.8 | 14.3 |
| E | *L. monocytogenes* | 4a | ATCC19114 | $1.43x10^8$ | 7.0 | 21.0 |
| F | *L. ivanovii* | | ATCC19119 | $1.73x10^8$ | 5.8 | 17.3 |
| G | *L. seeligeri* | | ATCC35967 | $2.1x10^8$ | 4.8 | 14.3 |
| H | *L. grayi* | | ATCC19120 | $2.1x10^8$ | 4.8 | 14.3 |
| I | *L. monocytogenes* | 1/2a | LMG16780 | $2.1x10^8$ | 4.8 | 14.3 |
| J | *L. monocytogenes* | 1/2b | LMG23190 | $2.1x10^8$ | 4.8 | 14.3 |

### 3.5.2 Pre-immunsation screening of mouse sera for existing anti-*Listeria* titre

Anti-*Listeria* activity of mouse sera tested in ELISA are shown in Figures 3.18 (a poorly responding mouse) and Figure 3.19 (a mouse responding well to immunisation). These are representational of poor response and very good response in two of the 15 mice. Both figures compare the anti-*Listeria* titre after three immunisations to the pre-immunisation titre of the same mouse.

The first seven mice immunised in this experiment (mice 1 through 7) were the group that received $10^7$ attenuated cells of *Listeria*. It is not clear if they have developed a lower titre of anti-*Listeria* antibody according to the ELISA than the mice immunised with $3x10^7$ attenuated cells. Mice 1, 2, 5, 7 and had weaker titres; four of these five received the lesser dose. This pattern may speculatively be due to the technique of the veterinary technician performing the procedure, for example the immunogen settling periodically out of adjuvant emulsion.

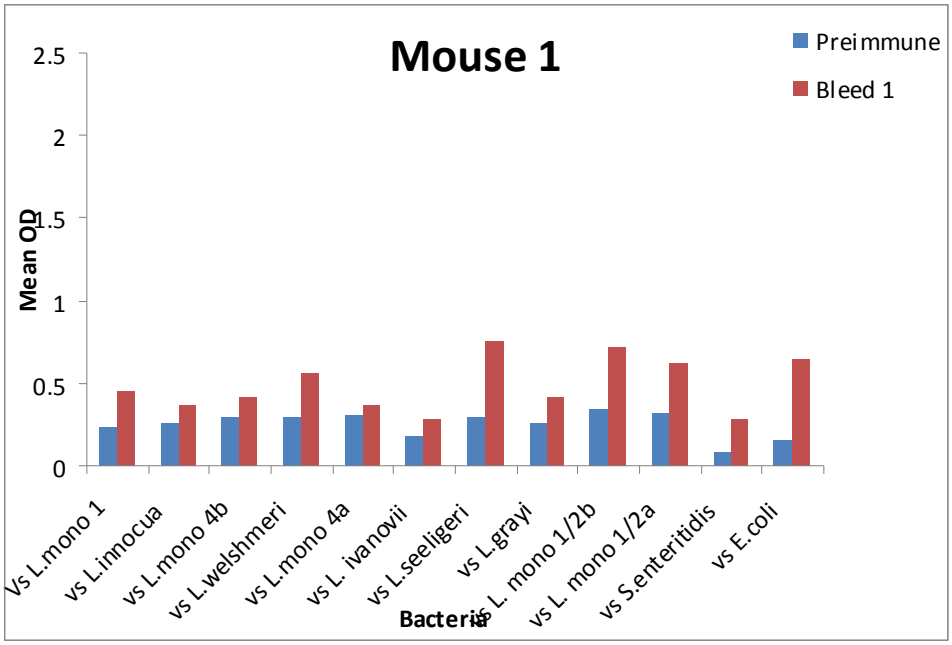



Figure 3.18: ELISA screening results of a typical poor response, in this case Mouse 1. Although anti-*Listeria* pAb titre is higher than the pre-immunisation serum, the difference is minimal.

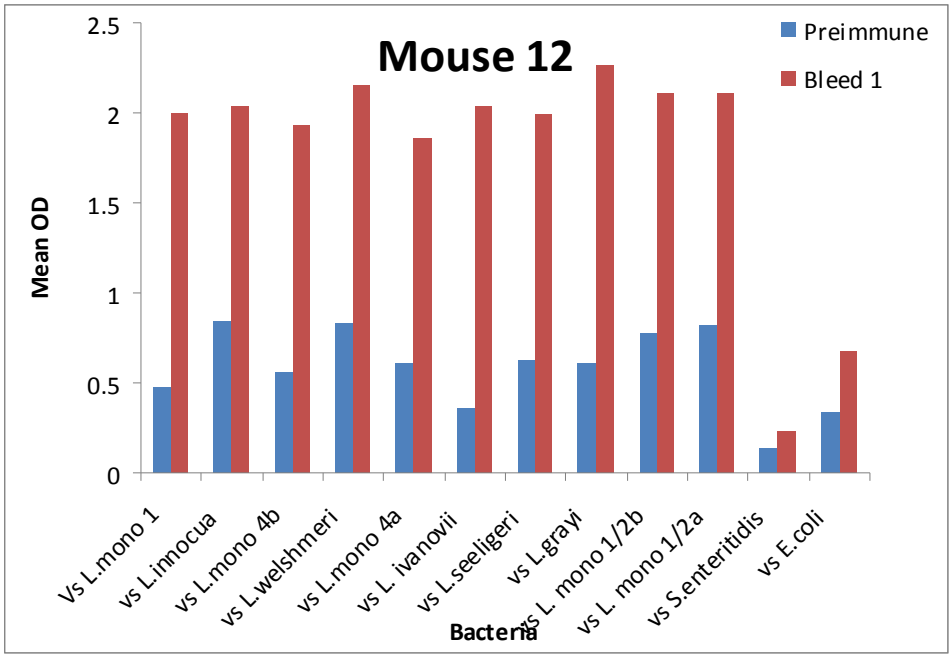



Figure 3.19: ELISA screening results of a typical good response, in this case of Mouse 12. Titre of anti-*Listeria* pAb after the first immunisation is far higher than that in Figure 3.18.

### 3.5.3 Hybridoma fusion

The first hybridoma fusion was successful in producing cells which, on switching to HAT medium, continued to survive and multiply. However upon screening none of these cells produced antibody specific to *Listeria* antigen as screened by indirect ELISA.

The second fusion was successful in producing anti-*Listeria* antibody secreting cells. In both cases, plates where cell density was high (plates 4 and 5) produced far more viable hybridoma cells than those that were more heavily diluted. The third fusion and the fourth fusion used multiple spleens and showed greatly increased numbers of positive wells on the screening plates. The results of these screenings are shown in section 3.5.4.

### 3.5.4 Hybridoma screening

ELISA screenings were run on 96 well microtitre plates mirroring the 96 well tissue culture plates. Any well with an absorbance value of 0.200 or above was taken as a potential positive well, i.e. a well containing antibody specific to *Listeria*. Figure 3.20 shows a typical screening plate result.

The first fusion did not produce any positive wells after the second screening (results not shown). The second fusion produced over 30 wells that were still positive after the second screening. The third fusion was carried out in the same way as the second, but four mouse spleens were used at once, resulting in a much higher number of hybridoma cells. The fourth fusion used two spleens to reduce the quantity of plates to a more maintainable number, but in all other respects it was the same as fusion 3. Cells isolated from the positive wells are listed in Tables 3.21 through 3.23, which are coloured for highlight: green for a low and inconclusive result (usually < 0.400); yellow for a weak but conclusive signal; and red for a strong signal (> 0.700) although these distinctions been adjusted relative to the plate and are relatively subjective. These are not totally inclusive of all positive wells collected and stored, but most of those not listed in these tables were only capable of detecting a single strain of

*Listeria*. There were up to 10 of these wells per fusion. Wells were considered negative if they either did not show any ELISA response above background, or it disappeared after the first screening.

Line F2-4C4 was dilution cloned several times, but did not successfully form a viable monoclonal line. Upon being isolated, it stopped expressing antibody after the first few days. After this, it would only express antibody when the clone died in the well. The F2-4C4 monoclonal anti-*Listeria* profile was confirmed as being identical to that before it was cloned. It was isotyped and confirmed as an $IgG_1$.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | Fail 0.114 | Fail 0.123 | Fail 0.186 | Pass 0.216 | Fail 0.147 | Fail 0.182 | Pass 0.25 | Pass 0.244 | Pass 0.247 | Fail 0.106 | |
| C | | Fail 0.0925 | Pass 0.334 | Pass 1.88 | Pass 0.259 | Pass 0.243 | Pass 1.13 | Fail 0.161 | Fail 0.171 | Fail 0.132 | Fail 0.144 | |
| D | | Fail 0.146 | Fail 0.114 | Fail 0.115 | Fail 0.188 | Pass 0.596 | Fail 0.145 | Fail 0.124 | Fail 0.131 | Fail 0.168 | Fail 0.11 | |
| E | | Pass 0.284 | Fail 0.133 | Fail 0.189 | Fail 0.113 | Fail 0.161 | Fail 0.102 | Fail 0.107 | Pass 0.301 | Pass 0.216 | Fail 0.152 | |
| F | | Pass 0.293 | Fail 0.148 | Fail 0.199 | Pass 0.395 | Fail 0.127 | Fail 0.145 | Pass 0.222 | Fail 0.122 | Fail 0.143 | Fail 0.0975 | |
| G | | Fail 0.152 | Fail 0.132 | Fail 0.122 | Pass 0.34 | Fail 0.141 | Pass 0.242 | Fail 0.171 | Pass 0.251 | Fail 0.108 | Fail 0.178 | |
| H | | | | | | | | | | | | |

Figure 3.20: Typical hybridoma screening plate: fusion 2, screening plate 4, first screening. Wells above 0.200 were considered of interest. If, upon the second screening, these wells were still positive and their 540 nm absorbance increased, they were selected for storage or cloning.

| Cell Well | A *L. mono 1* | B *L. innocua* | C *L. mono* 4b | D *L. welshimeri* | E *L. mono 4a* | F *L. ivanovii* | G *L. seeligeri* | H *L. grayi* | I *L. mono 1/2b* | J *L. mono 1/2a* | *E. coli* | *S. enter* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F2-4C4 | 0.073 | 2.323 | 1.427 | 1.998 | 1.164 | 1.781 | 1.563 | 0.460 | 1.964 | 1.501 | 0.061 | 0.060 |
| F2-4D6 | 0.067 | 0.401 | 0.058 | 2.330 | 1.307 | 0.098 | 0.065 | 0.198 | 0.080 | 0.055 | 0.055 | 0.070 |
| F2-5E2 | 0.054 | 0.318 | 0.084 | 0.210 | 0.130 | 0.370 | 0.050 | 0.077 | 0.092 | 0.124 | 0.046 | 0.047 |
| F2-5B4 | 0.198 | 0.345 | 0.493 | 0.478 | 0.539 | 0.592 | 0.528 | 0.491 | 0.448 | 0.782 | 0.324 | 0.260 |
| F2-5E5 | 1.613 | 0.565 | 1.367 | 0.322 | 0.252 | 0.273 | 0.336 | 0.196 | 1.896 | 1.987 | 0.129 | 0.083 |
| F2-4E10 | 0.404 | 0.902 | 0.116 | 0.689 | 0.717 | 1.180 | 0.713 | 0.060 | 1.370 | 0.664 | 0.054 | 0.054 |

Table 3.21: Selected positive isolates from second hybridoma fusion; supernatant tested by indirect ELISA against a microtitre plate coating of all *Listeria*. *E. coli* and *S. enteriditis* were used as negative controls.

| Cell Well | A *L. mono 1* | B *L. innocua* | C *L. mono 4b* | D *L. welshimeri* | E *L. mono 4a* | F *L. ivanovii* | G *L. seeligeri* | H *L. grayi* | I *L. mono 1/2b* | J *L. mono 1/2a* | *Kurthia zopfii* | *Br. laterosporus* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F3-4E3 | 0.069 | 0.070 | 0.117 | 0.110 | 0.120 | 0.118 | 0.127 | 0.160 | 0.121 | 0.151 | 0.145 | 0.170 |
| F3-3H6 | 0.063 | 0.195 | 0.124 | 0.485 | 0.089 | 0.252 | 0.344 | 0.057 | 0.268 | 0.136 | 0.053 | 0.088 |
| F3-3B10 | 0.058 | 1.156 | 0.092 | 2.929 | 0.059 | 0.883 | 0.064 | 0.163 | 0.179 | 0.060 | 0.055 | 0.158 |
| F3-5A7 | 0.093 | 1.034 | 0.327 | 1.641 | 0.219 | 1.137 | 0.114 | 0.438 | 0.483 | 0.064 | 0.050 | 0.851 |
| F3-5A12 | 0.179 | 2.694 | 0.080 | 6.000 | 0.483 | 0.590 | 0.189 | 0.072 | 0.735 | 0.193 | 0.063 | 0.093 |
| F3-4A8 | 0.117 | 0.134 | 0.106 | 0.083 | 0.123 | 0.101 | 0.061 | 0.090 | 0.121 | 0.100 | 0.051 | 0.051 |
| F3-6H4 | 0.560 | 2.994 | 0.154 | 6.000 | 0.965 | 0.958 | 0.341 | 0.219 | 1.046 | 0.366 | 0.199 | 0.207 |

Table 3.22: Selected positives from fthird usion, supernatants as before assayed by ELISA. As this fusion was done after the first food and broth tests, negative controls were changed to *K. zopfii* and *Br. laterosporus*.

| Cell<br>Well | A<br>*L. mono 1* | B<br>*L. innocua* | C<br>*L. mono 4b* | D<br>*L. welshimeri* | E<br>*L. mono 4a* | F<br>*L. ivanovii* | G<br>*L. seeligeri* | H<br>*L. grayi* | I<br>*L. mono 1/2b* | J<br>*L. mono 1/2a* | *Kurthia*<br>*zopfii* | *Br.*<br>*laterosporus* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F4-1F2 | 0.559 | 0.605 | 0.148 | 0.324 | 1.990 | 1.392 | 0.203 | 0.324 | 0.515 | 0.101 | 0.056 | 0.069 |
| F4-2D2 | 0.652 | 0.689 | 0.197 | 0.208 | 2.596 | 1.662 | 0.223 | 0.108 | 0.510 | 0.125 | 0.076 | 0.091 |
| F4-2H6 | 0.112 | 0.122 | 0.070 | 0.083 | 0.093 | 0.072 | 1.842 | 0.070 | 0.076 | 0.069 | 0.069 | 0.078 |
| F4-3D2 | 0.652 | 0.057 | 0.103 | 0.053 | 0.129 | 0.051 | 0.294 | 0.046 | 0.522 | 0.132 | 0.048 | 0.042 |
| F4-3D6 | 1.896 | 0.790 | 0.382 | 0.218 | 2.911 | 2.445 | 0.647 | 0.207 | 1.042 | 0.252 | 0.125 | 0.159 |

Table 3.23: Selected positive isolates from fourth and final hybridoma fusion; supernatant tested by indirect ELISA against a microtitre plate coating of all *Listeria*. *K. zopfii* and *Br. laterosporu*s. were used as negative controls.

The presence of IgM-expressing originator B-cells in heavily immunised mice was not expected. Unlike in previous fusions the positives from fusion 4 were isotyped before cloning. All but F4-2B12 were IgM kappa. Table 3.24 shows the results of the isotyping test. Because of this predominance of IgM lines, fusion 4 positives were re-screened with an anti-mouse γ-chain specific secondary antibody rather than general anti-mouse heavy and light chain. The results in Table 3.25 show the ELISA anti-*Listeria* IgG-only binding of the antibody in these supernatants.

It is not known if any of the positive wells in fusions 2 and 3 were also IgM, but F2-4C4, the only other line that was cloned, was $IgG_1$ and still had specificity for almost all *Listeria*.

500 ml of F4-1F2-4C8 was purified using Pierce T-Gel, quantified at 280 nm using the IgM molar extinction coefficient of 1.18[36]. It was then coated at 20 μg $ml^{-1}$ and blocked as usual. KPL BacTrace labelled with horseradish peroxidise was used as detected antibody at 2 μg $ml^{-1}$ in 3% (w/v) casein in PBS. Table 3.40 shows the ELISA results using this method.


Figure 3.24: Isotyping of selected fusion 4 well supernatants. As the isotyping kit used was ELISA based, it allowed multiple Ab heavy and light chains to be detected if present. The red wells show a positive result (> 0.200).for a discrete heavy chain or light chain.

| | L. mono 1 | L. innocua | L. mono 4b | L. welshimeri | L. mono 4a | L.ivanovii | L.seeligeri | L. grayi | L. mono 1/2b | L. mono 1/2a | Blank |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **F4-1C10** | 0.086 | 0.086 | 0.087 | 0.081 | 0.089 | 0.092 | 0.083 | 1.199 | 0.087 | 0.086 | 0.078 |
| **F4-1F2** | 0.052 | 0.051 | 0.052 | 0.049 | 0.051 | 0.054 | 0.049 | 0.701 | 0.047 | 0.051 | 0.047 |
| **F4-2H4** | 0.050 | 0.047 | 0.049 | 0.049 | 0.050 | 0.047 | 0.043 | 0.049 | 0.047 | 0.048 | 0.047 |
| **F4-2H6** | 0.050 | 0.049 | 0.047 | 0.050 | 0.050 | 0.048 | 0.872 | 0.046 | 0.045 | 0.046 | 0.047 |
| **F4-1H5** | 0.065 | 0.058 | 0.055 | 0.055 | 0.060 | 0.060 | 0.054 | 0.055 | 0.049 | 0.055 | 0.051 |
| **F4-2D2** | 0.055 | 0.047 | 0.047 | 0.053 | 0.047 | 0.054 | 0.046 | 0.046 | 0.046 | 0.044 | 0.045 |
| **F4-3D2** | 0.234 | 0.062 | 0.070 | 0.057 | 0.077 | 0.058 | 0.115 | 0.047 | 0.168 | 0.080 | 0.042 |
| **F4-3E6** | 0.058 | 0.056 | 0.053 | 0.053 | 0.053 | 0.057 | 0.048 | 0.040 | 0.049 | 0.045 | 0.047 |

Table 3.25: Fusion 4 positive supernatants screened by ELISA with anti-mouse anti-γ as a secondary. This confirms that most of the activity in the previous ELISA screenings was due to IgM Abs.

| | *L. mono 1* | *L. innocua* | *L. mono 4b* | *L. welshimeri* | *L. mono 4a* | *L. ivanovii* | *L. seeligeri* | *L. grayi* | *L. mono 1/2b* | *L. mono 1/2a* | *K. zopfii* | *Br. laterosporus* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.062 | 0.195 | 0.234 | 0.574 | 0.173 | 1.574 | 0.282 | 0.703 | 0.775 | 0.135 | 0.082 | 0.271 |
| | 1.019 | 0.194 | 0.237 | 0.380 | 0.093 | 1.584 | 0.238 | 0.764 | 0.408 | 0.096 | 0.068 | 0.112 |
| **Mean** | 1.041 | 0.194 | 0.236 | 0.477 | 0.133 | 1.579 | 0.260 | 0.733 | 0.591 | 0.115 | 0.075 | 0.192 |
| **SD** | 0.030 | 0.000 | 0.003 | 0.137 | 0.057 | 0.007 | 0.031 | 0.043 | 0.259 | 0.028 | 0.010 | 0.112 |

Table 3.26: ELISA detection of all *Listeria* strains with F4-1F2-4C8 as a primary Ab with KPL as capture Ab.

## 3.6 Broth development for food testing

### 3.6.1 Evaluation of SLB with foodstuffs

The spread plates reported that the mean number of *Listeria* CFUs being inoculated into the foods, which was calculated to be 3 CFU in 1 ml of inoculum, was actually 1.9 for *L. innocua*, 2.7 for *L. monocytogenes* serovar 4b, 21.6 for *L. monocytogenes* serovar1/2b, 32.1 for *L. monocytogenes* serovar 1/2a, and 10.9 for *L. seeligeri*.

As evident from CLISA (Table 3.27), the initial test revealed some weaknesses in the assay, most notably substantial background signal. Although this could have been due to existing *Listeria* contamination in the foods, for it to be present in all of the samples was very unlikely and more likely due to the antibody cross-reacting with other bacteria within the food or with the food matrix itself.

In the case of pate, infant formula and capsicum, high backgrounds obscure the detection of *Listeria*, though it can be seen. *Listeria* cultured in beef mince and haddock did not appear to have been detected at all, with all results not deviating significantly regardless of inoculum, and no high background. This was most probably due to the presence of high numbers of bacteria inhibitory to *Listeria* able to thrive in SLB, and this view is supported by the fact that mince and haddock had the highest APC counts (Table 3.28).

Both OAA and Rapid L mono plates reported positives where there was no *Listeria* inoculum. Colonies of these positives were plated into nutrient agar and cultured overnight at 30°C then tested with latex agglutination where they were all certified as not *Listeria*. The ISO method, and Rapid L. mono in general, suffered less from these false positives but in the case of beef mince there were still issues.

The organism responsible for the false positives forms fairly-well growing colonies of the following traits: round, convex, shiny, white (where on non-chromogenic agar), or green (on OAA). They grow considerably less well on Rapid L mono but still grow

more rapidly on it than some *Listeria* strains (for example *L. monocytogenes* serovar 1, or *L. seeligeri*). Colonies produced a halo of lecithinase activity, were gram positive, and had a similar appearance to *Listeria* under the microscope (moderate sized rods). It was indistinguishable from *Listeria* except in that did not grow on Oxford agar, was strictly aerobic, and that immunological testing (seriological latex agglutination, KPL BacTrace) did not identify it as *Listeria*. From its morphological traits it is most likely that it is a strain, or mixture of strains, of *Bacillus circulans* although this could not be confirmed.

| | Mean RLUs | Innoculum | | | | |
|---|---|---|---|---|---|---|
| **Food** | **Food Only** | ***L. mono* 4b** | ***L. mono* 1/2a** | ***L. mono* 1/2b** | ***L. innocua*** | ***L. seeligeri*** |
| Pate | 24688 | 10834.5 | 38645.5 | 16636.2 | 53034 | 10056.5 |
| Infant Formula | 24175.5 | 41684.5 | 18031 | 11736.5 | 31032.5 | 26645 |
| Beef mince | 5489.5 | 6453 | 5963 | 5916.2 | 4738 | 4018.5 |
| Capsicum | 14635.5 | 35163.5 | 8056.5 | 5843 | 20200.5 | 23494 |
| Haddock | 8201 | 8703 | 8098 | 7986.5 | 8780 | 9482 |

Table 3.27: CLISA results for initial *Listeria* food test, using KPL BacTrace as both detection and capture Ab.

| **Food** | **Mean CFU (per plate)** | **Dilution Factor** | **Food CFU/g** |
|---|---|---|---|
| Pâté | 1 | 1:10 | $10^3$ |
| Infant Formula | 1 | 1:10 | $10^3$ |
| Beef Mince | 190 | 1:100 | $1.9x10^6$ |
| Capsicum | 1 | 1:10 | $10^3$ |
| Haddock | 173 | 1:1000 | $1.70x10^7$ |

Table 3.28: Aerobic plate counts of foods prior to incubation.

As a reliable basis of comparison, the chromogenic agar (both using SLB and the ISO method) was able to detect the presence of *Listeria* even with only a few viable cells. If the CLISA were successful, it should have RLU counts significantly higher than background where the agar was found to have *Listeria* colonies.

| | Chromogenic Agar Results | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | From ISO 24h | | From ISO Fraser 1 | | From SLB | | From SLB then Fraser | |
| Food | OAA | RAPID | OAA | RAPID | OAA | RAPID | OAA | RAPID |
| Pâté | - | + | NG | NG | + | + | +++ | + |
| Infant Formula | NG | NG | NG | NG | +++ | +++ | +++ | -- |
| Beef Mince | NG | NG | ++++ | NG | NG | + | NG | NG |
| Capsicum | NG | NG | NG | NG | + | + | NG | NG |
| Haddock | NG | NG | NG | NG | NG | + | NG | NG |

Table 3.29: Chromogenic agar results for ISO and SLB methods (original food). Positive growth (where colonies exhibited the colour and/or haloes expected) is marked between + (some colonies) to ++++ (confluent plate). Significant negative growth, those colonies that did not fit the chromogenic and/or morphological profile, is marked with – symbols. NG denotes no noticeable growth on the plate of any colonies.

### 3.6.1 Identification of bacteria in food tests using SLB

Organisms at this point were taken from the chromogenic agar rather than the food itself, as it was considered better to deal with false-positive bacteria first before looking at the more complex problem of finding inhibitory strains capable of growing in SLB. No easy way existed to characterise these false positive bacteria, such as an API test. The most common contaminating species were the most easy to spot morphologically because of particularly noticeable colony shapes that were radically different to *Listeria* and most other gram positives. All were gram positive rods, all were capable of growth at room temperature and at 45°C (but not 4°C) all were γ-haemolytic (did not effect blood agar). Growth was particularly rapid on rich media such as BHI Agar, where noticeable growth would appear within six hours at 37°C. After assessment by Microgen Bacillus ID, it was determined that these false positives were most likely of the *Bacillus* family or closely related families such as *Brevibacillus*. The *Bacillus* species is extremely diverse and even different strains of the same species proved in the food testing described to have different biochemical profiles and growth conditions. *Listeria* itself is a member of the *Bacillae*, so it would not be unreasonable to expect to find *Bacillus* strains with antibiotic resistances and enzyme expression similar or identical to *Listeria*.

Those colonies isolated from mince, fish and pâté plates were regular rods, short but not as short as *Listeria*, and were oxidase and catalase negative. They were strictly aerobic, non-haemolytic and did not appear to have visible endospores when examined by microscope. They were also capable of growth at 45°C and not at 4°C. On being allowed to grow at room temperature on nutrient agar or BHI agar for several days, streaks formed a feather pattern or "medusa head" rhizome cream-coloured colonies. From these features, particularly the latter, it is most likely these are of the *Kurthia* species.

The bacteria isolated from formula milk were initially presumptively identified as *Corynebacterium* due to racquet-shaped cells and their strictly aerobic nature. This identification became increasingly unlikely as the work continued. The distinct shape of the cell may have been due to an endospore, but the anaerobic *Clostridium* was

immediately dismissed, and *Bacillus* was less suspected due to it being oxidase negative and catalase positive, as well as the majority of species being aerobic and β-haemolytic. It also did not form into chains, and produced small off-white round colonies on agar very similar to *Listeria*, and almost identical colour change on OAA (glucosidase positive, lecithinase positive). On this agar it was indistinguishable from *Listeria* except by latex agglutination.

Counter-intuitively, it should be noted that infant formula milk is not claimed to be sterile and certainly proved as not being sterile during the tests. Having been pasteurised rather than ultra-heat treated to prevent the destruction of nutrients, it could potentially harbour spores of common milk contaminants such as *Bacillus*, making spore-formers more likely as candidates as to the identity of the above unknown bacterium. It is unlikely that any member of the *Corynebacterium* group could survive pasteurisation as it does not form spores. It was most probably *B. circulans* because of its round colonies (uncharacteristic for most *Bacillus*), although Microgen Bacillus ID could not confirm this.

Other species encountered were small, inhibited colonies on Rapid L Mono plates which were tetrads of gram positive cocci, catalase and oxidase positive, γ-haemolytic, and strictly aerobic. When plated on BHI agar they formed small, round, convex yellow colonies. These were presumptively identified as the common *Micrococcus luteus*.

No gram-negative bacteria were encountered from the plates or incubated foods at this stage. SLB proved sufficient to prevent the growth of this very large group. The only possible exception to this was the Pseudomonads, the presence of which was identified by their ability to grow when colonies were picked from LB-agar and streaked on cetrimide agar.

### 3.6.1 Antibiotic susceptibility testing of *Listeria*

This level of antibiotic testing aimed to identify concentrations and combinations of antibiotics that would permit *Listeria* growth. Table 3.30 shows the result of rudimentary agar testing with cephalothin, acriflavine, nitrofurantoin (NFT) and cefoxitin.

In the agar tests, cephalothin at 3 mg $l^{-1}$ inhibited the other gram positive rods tested but proved fully inhibitory for *Listeria*. Acriflavine affected mostly the "*Corynebacterium*" (*Bacillus*) from infant formula although it did inhibit the *Listeria* slightly. Cefoxitin inhibited *Micrococcus* and *Kurthia* from foods and did not affect *Listeria* except at the highest dose; however it did not affect a suspected *Streptococcus* or *E. faecalis* at all. Cephalothin completely inhibited *Listeria* even at a low dose and was therefore eliminated from potential formulations.

Nitrofurantoin (NFT) had a very powerful effect on all cocci at 16 mg $l^{-1}$ and did not affect *Listeria*. Given the formidable resistance *E. faecalis* to most antibiotics and its powerful suppression of *Listeria* growth, this makes it a good choice for any broth formulation.

However, the affect of these antibiotics in broth (Figure 3.31) did not in any way match those on agar, particularly in the case of the cephalosporins. For this reason testing on agar was discontinued.

To supplement the NFT, ceftsulodin and ceftazidime were both tested as a replacement for cefoxitin, first against *L. monocytogenes* serotype 4b alone (Figure 3.32). Neither of these antibiotics were particularly inhibitory to *L. monocytogenes*, even at high levels.

| Organism Tested | Original SSLB | Cephalothin 3 ug | Acriflavine 5ug | Cefoxitin 4ug | Cefoxitin 8ug | Cefoxitin 20ug | 8ug Cefoxitin 8ug NFT | 4ug Cefoxitin 16ug NFT |
|---|---|---|---|---|---|---|---|---|
| *L.mono 4b* | ——— | NG | ——— | ——— | ——— | NG | ——— | ——— |
| *Streptococcus* | ——— | ——— | ——— | ——— | ——— | ——— | ——— | ——— |
| *E.Faecalis* | ——— | ——— | ——— | ——— | ——— | ——— | ——— | ——— |
| *Coryne* | ——— | NG | NG | ——— | ——— | NG | ——— | ——— |
| *Micrococcus* | ——— | NG | ——— | NG | NG | NG | NG | NG |
| *Kurthia* | ——— | NG | ——— | NG | NG | NG | NG | NG |

Table 3.30: Antibiotic testing (agar) results. This shows the result of rudimentary agar testing with cephalothin, acriflavine, nitrofurantoin (NFT) and cefoxitin. NG denotes no observable growth. nitrofurantoin does not significantly alter detection of *Listeria*, while cefoxitin prevents the detection (and therefore growth) of *Listeria* even at low doses.

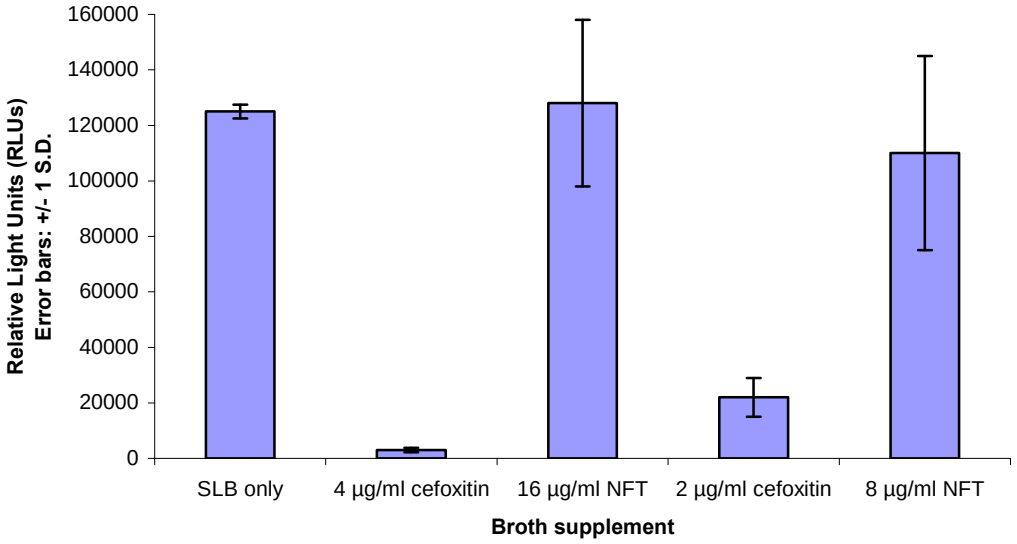



Figure 3.31: *Listeria* inhibition by cefotixin as a selective supplement in broth. The effect of cefoxitin did not in any way match those on agar, particularly in the case the cephalosporins, and was very inhibitory even at small concentrations.

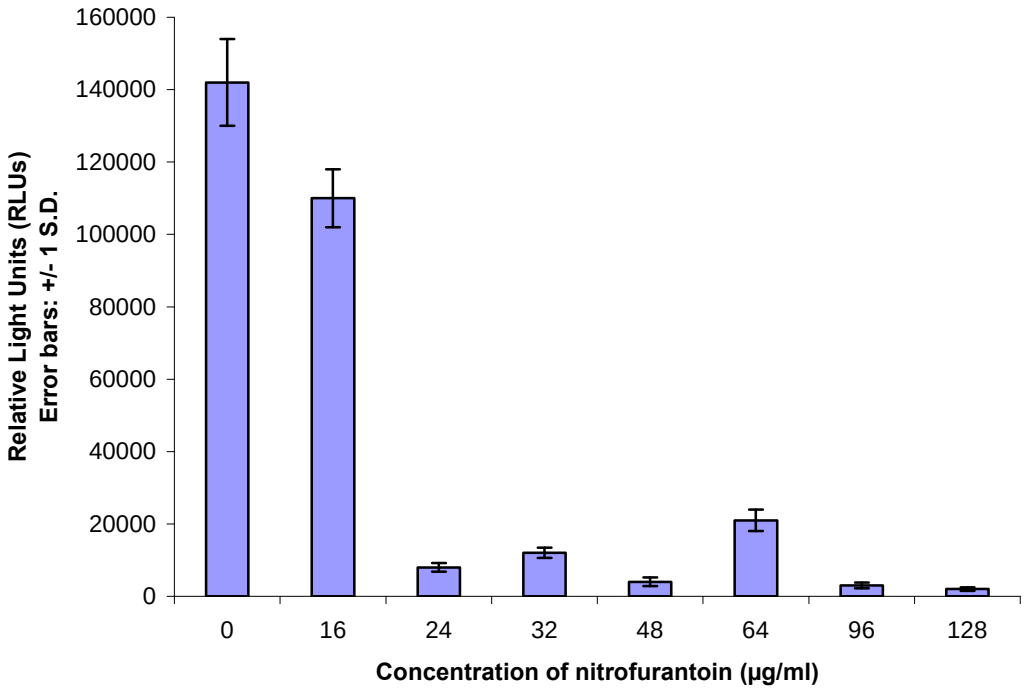



Figure 3.32: the effect of increasing levels of nitrofurantoin on *Listeria* growth. 16 µg $ml^{-1}$ does seem to slightly inhibit *Listeria*. All concentrations greater than this have a very marked and profound inhibitory effect. From these results a value of 12 µg $ml^{-1}$ in the broth formulation was settled upon as a compromise between effectiveness against cocci and inhibition of *Listeria*.

When tests on a single foodstuff were repeated using these antibiotics (cod fillet), both were found to eliminate the problem of *Listeria* inhibition in SLB at 2 μg $ml^{-1}$, at least at *Listeria* inoculum of around 1000 CFU or more (Figure 3.33). However, in the case of the cefsulodin formulation, it was discovered that the BHI agar plates streaked using the incubated food were stained dark green; this was due to the presence of *Pseudomonas aerugenosa*, which produces the blue-green pigment pyocyanin that also happens to be inhibitory to other bacteria[37]. *P. aerugenosa* was confirmed by growth and production of vivid yellow fluorescent pigment (fluorescein) when streaked on cetrimide agar. It was doubly confirmed by API 20NE. *P. luteola* was also isolated on the cetrimide agar and identified by API 20NE. The presence of *Pseudomonas* could prove inhibitory to *Listeria*, although it was not seen in this case, where the original inoculum of *Listeria* is low and/or stressed.

These particular cultures were chosen and the experiment excluded *Kurthia* and *Corynebacterium* because these latter two had already been observed to cross-react very significantly with the KPL BacTrace antibody. This makes it impossible to use the CLISA assay to quantify the level of *Listeria* when mixed with these organisms using this antibody.

From the CLISA results, *Micrococcus* (most probably *M. luteus* from its yellow colour) does not interfere with *Listeria* growth and neither does the suspected *Streptococcus*. In contrast, both *E. faecalis* and *P. luteola* almost completely suppressed *Listeria* growth, with the latter reducing the signal to background levels (Figure 3.34).

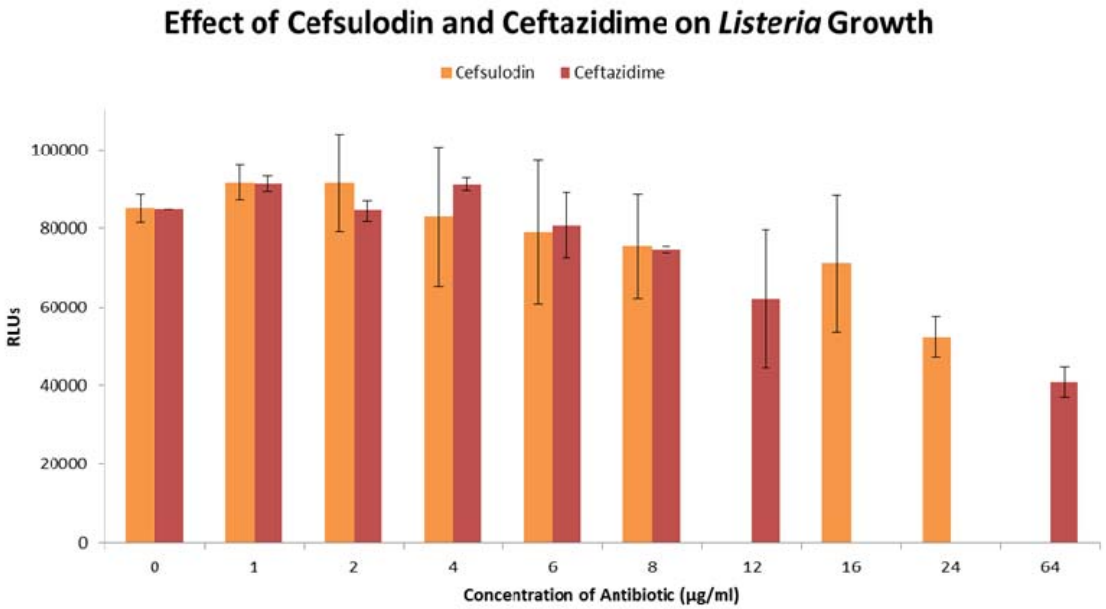



Figure 3.33: Effect of cefsulodin and ceftazidime on *Listeria* growth, measured by CLISA. (Note that combinations of 12 µg $ml^{-1}$ and 64 µg $ml^{-1}$ for cefsulodin and 16 µg $ml^{-1}$ and 24 µg $ml^{-1}$ for ceftazidime were not tested. These are not representative of zero RLUs.) Error bars display ±1 standard deviation.

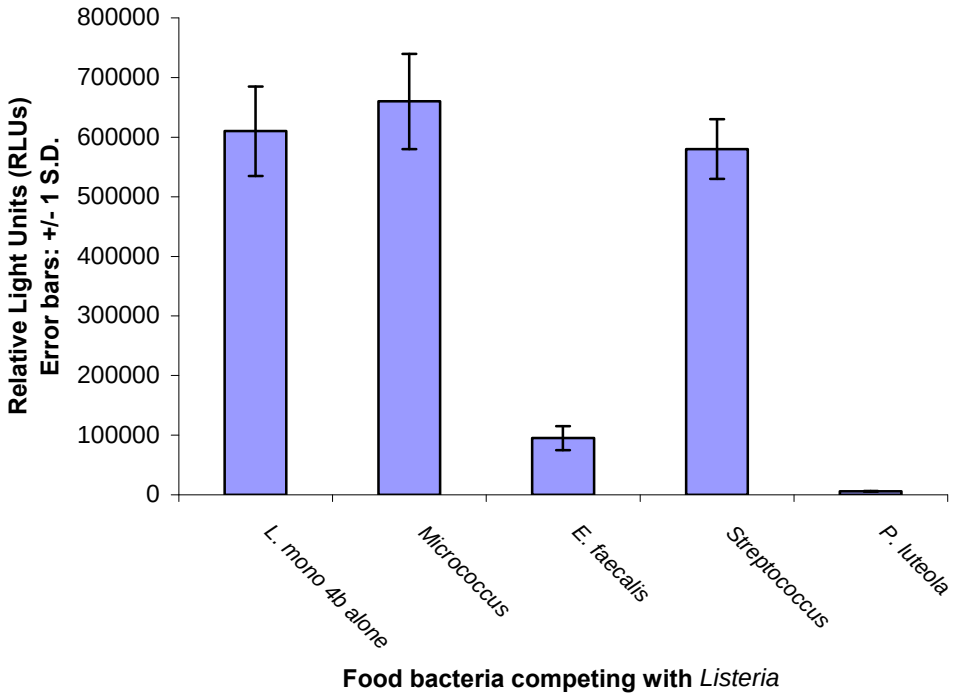



Figure 3.34: Effect of competing organisms on *Listeria* growth and detection by CLISA in original SLB. *E. faecalis* and *P. luteola* are observed to heavily suppress *Listeria* growth.

### 3.6.4 Investigation of ceftazidime as *Listeria* selective component

The effect of the modification of SLB with the addition of ceftazidime was asecertained by retesting "problem" foods – namely yoghurt and infant formula milk – with the modified broth (mSLB). Ceftazidime proved the most effective antibiotic in terms of suppressing other microflora (particularly *Pseudomonas*) out of the cephalosporins. It is a common component of *Listeria* selective agars.

The modified broth used with yoghurt successfully detected *Listeria* after a touch-colony inoculation (approximately 1000 CFU) – see Figure 3.35. Figure 3.36 shows two potentially foods, unsmoked cod fillet and beef mince after inoculum with >1000 CFU and 20 hour incubation.

Detection of all *Listeria* is very pronounced for most strains, but weaker with *L. monocytogenes* serotypes 1 and 1/2b, and *L. ivanovii*, especially in beef mince. In this food *L. monocytogenes* serotype 1/2a was not detected at all. This suggested that inhibitory bacteria common in beef mince were responsible for inhibition and that the modified broth was still not fully selective enough for purpose. Following these tests the level of acriflavine was increased to 1.5 μg $ml^{-1}$, as it was the only antibacterial compound in the broth that was not close to its *Listeria* inhibitory dose.

It should also be noted that endogenous *L. monocytogenes* and *L. innocua* were discovered twice on cod fillets from the same outlet, different batch, which may suggest that *Listeria* is a fairly common contaminant of raw fish products. These bacteria were confirmed by API test.

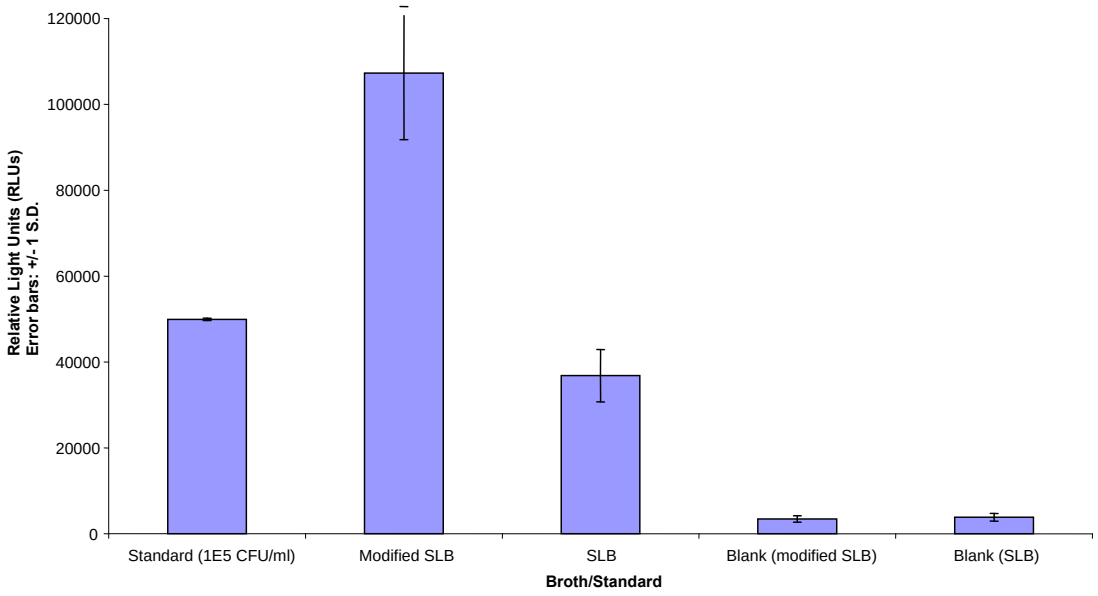



Figure 3.35: CLISA comparison of SLB vs SLB with the addition of ceftazidime (mSLB), in natural yoghurt (approximately 1000 CFU *L. monocytogenes* serotype 4b inoculum). *Listeria monocytogenes* is apparently still capable of growing to significant levels in yoghurt with original SLB, with a high inoculum, but possibly not with a much smaller or stressed one. This may be due to its expression of unknown factors that may suppress the growth of endogenous spore-forming gram positive *Bacillus*. In realistic inoculum such as those likely to be found in *Listeria*-contaminated food however, due to number and rate of growth, other contaminating organisms such as *Bacillus* would completely outgrow and suppress any *Listeria*. The modified SLB was far superior in performance in any case. Error bars display ±1 standard deviation.

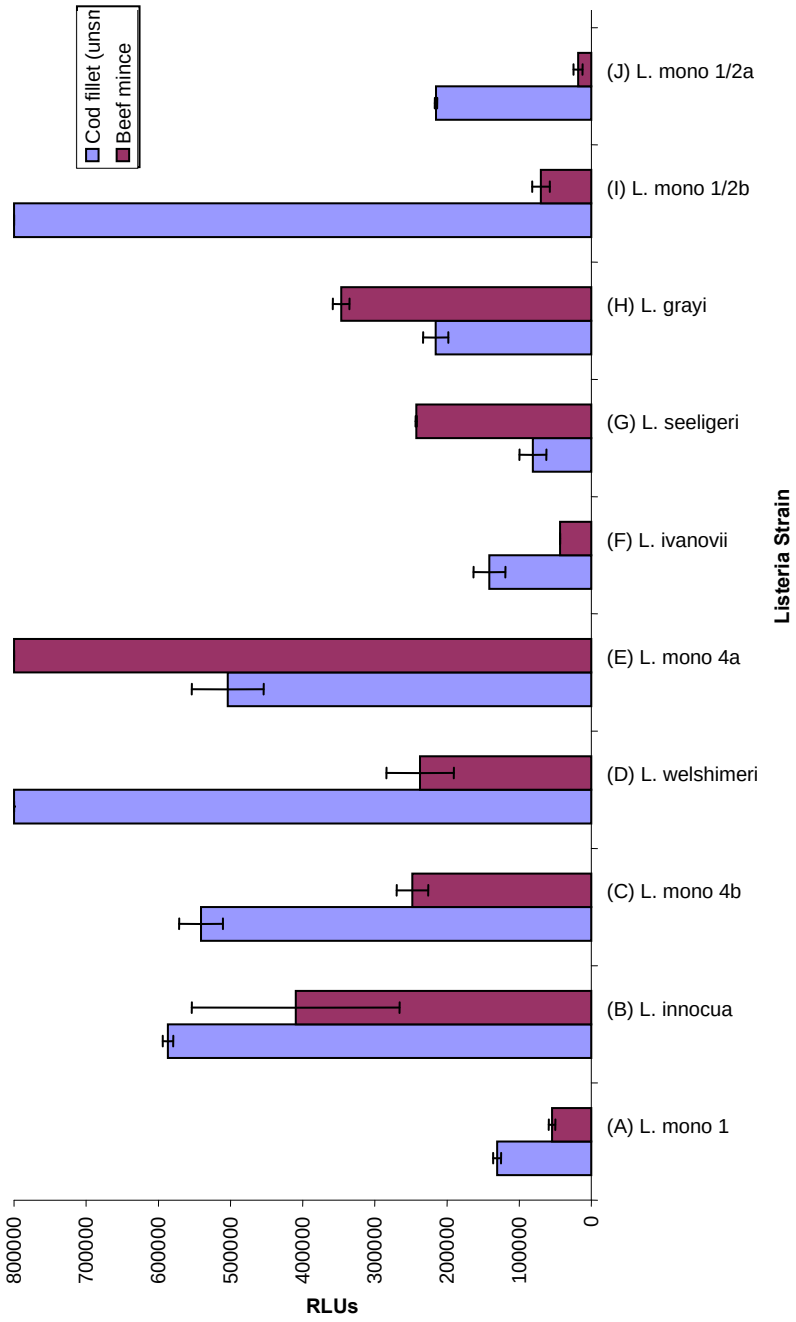



Figure 3.36: CLISA of foods spiked with Listeria strains. Error bars display ±1 standard deviation.

### 3.6.5 Modification of SLB for suppression of bacteria in infant formula milk

Although a combination of acriflavine, nitrofurantoin and ceftazidime were sufficient for the foods tested in section 3.6.4, the growth of *Bacillus* species in milk products, particularly infant milk powder, remained a problem that was approached by again altering the broth composition.

Increasing acriflavine levels had little or no effect on *Bacillus* growth. At 5 mg $l^{-1}$ and above, it suppressed *L. seeligeri* enough so that even at low inoclum (1000 CFU), *Bacillus* would still be able to reach sufficient numbers to prevent *Listeria* growth (Table 3.37). Increasing lithium chloride and nalixidic acid in the broth had no discernable effect on either *Listeria* or competing bacteria (results not shown).

Chloramphenicol was also tested, but all concentrations down to 0.1 mg $l^{-1}$ in mSLB completely killed (rather than suppressed) all strains of *Listeria*; it did not recover and grow when streaked onto Rapid L mono or OCLA agar, even from 1000 CFU inoculum. It did not appear to have any lasting effect on *Bacillus* at any of the concentrations tested (up to 4 mg $l^{-1}$): large irregular colonies still appeared on the plates when loops of the broth were streaked on them.

Sulfamethoxazole, a sulphonamide, had no measurable effect on *Listeria* or *Bacillus* even at very high doses (128 mg $l^{-1}$). Higher concentrations could not be tested due to its insolubility in water and the necessity of adding greater and greater quantities of solvent (DMSO) to the broth, the effects of which were unknown.

Cefotetan was found to have much more pronounced bacteriocidal effect on *Bacillus*, and at 7 mg $l^{-1}$ was capable of completely eliminating *Bacillus* contamination while having negligible effect on *L. ivanovii* (Table 3.38).

Fosfomycin and fusidic acid were also scheduled to be tested, but the success of cefotetan meant that that they were not necessary to investigate.

| Acriflavine Mg $l^{-1}$ | Rapid L mono | |
|---|---|---|
| | *Listeria* | *Bacillus* |
| 1.5 | ++ | NG |
| 3 | ++ | NG |
| 5 | NG | ++++ |
| 10 | NG | ++++ |
| 15 | NG | ++++ |
| 20 | NG | ++++ |
| 25 | NG | ++++ |

Table 3.37: Effect of increasing levels of acriflavine in mSLB with infant formula (Rapid L mono agar).

| Cefotetan (mg $l^{-1}$) | *L. mono* 4b | *L. ivanovii* | *Bacillus* (All spp.) |
|---|---|---|---|
| 0 | NG | NG | ++++ |
| 3 | NG | NG | ++ |
| 5 | + | NG | + |
| 7 | +++ | +++ | NG |
| 10 | + | NG | NG |
| 12 | NG | NG | NG |

Table 3.38: Effect of cefotetan concentration on *Listeria* and *Bacillus* growth. *Listeria* fails to grow at lower levels of cefotetan because of the inhibitory effect of *Bacillus* species from the milk. Higher levels are inhibitory to *Listeria* as well.

### 3.6.6 *Listeria* growth rate in SLB and modified SLB, measured by CLISA

Upon testing broth modifications on infant formula milk, it soon became clear that further modification would be necessary due to very heavy *Bacillus* growth. The sheer quantity of *Bacillus* in some foods made them impossible to assay as long as all but minimal *Bacillus* growth was supported by the broth. This was particularly noticeable in infant formula milk, and less so in raw meats and yoghurt.

The diversity of *Bacillus* strains meant that their effect (when present) on *Listeria* growth was essentially random, producing highly variable immunoassay results. This resulted in assay failing to detect *Listeria* or detecting it only intermittently.

Also, KPL BacTrace antibody used in the immunoassay was also highly variable in its detection of *Bacillae*. Some strains had a very high ELISA or CLISA cross-reactivity, while others were poorly or not detected. This did not appear to be consistent across a single species and may well be strain dependent.

*Listeria* growth in Selective *Listeria* Broth and modified Selective *Listeria* Broth (without cefotetan) was quantified in pure cultures to directly compare both broths and identify any *Listeria* strains that would be problematic. Figures 3.39 through 3.48 show growth of each of the ten *Listeria* strains cultured between 16 and 24 hours measured by CLISA. Error bars show two standard deviations from the mean. Cefotetan-modified broth was not used in these growth rate studies.

It should be noted that these graphs show the concentration of *Listeria* antigen specific to KPL BacTrace present in the broth, and not numbers of *Listeria* cells or even mass of *Listeria*. It is an assumption that there should be a correlation between concentration of antigen(s) and *Listeria* numbers, although there is currently no reason why this should not be the case.

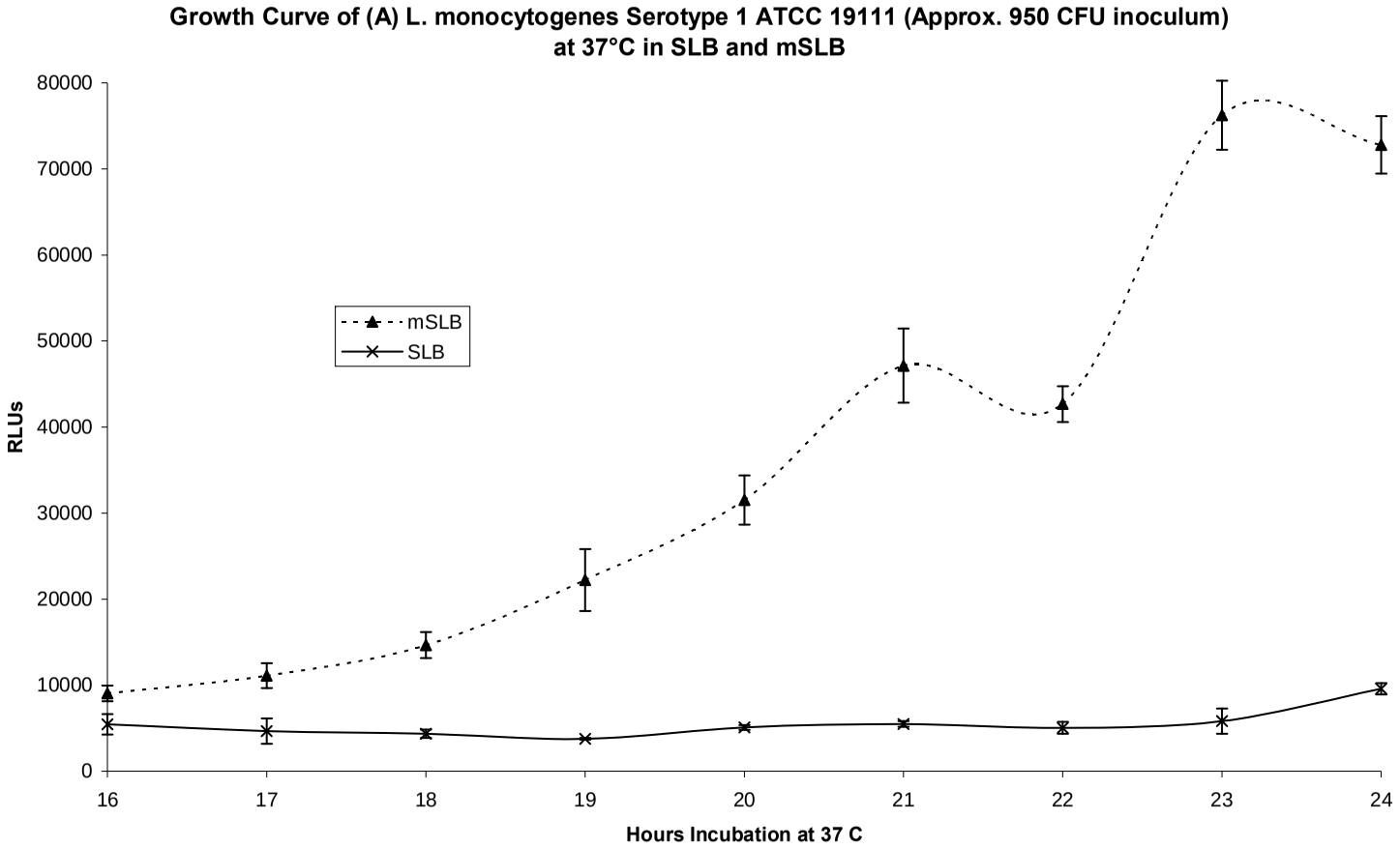



Figure 3.39: *L. monocytogenes* shows gradual increase, peaking at 21 hours. There is a dip in *Listeria* antigen CLISA detection at 22 hours. The reason for this is unknown. This was followed by another increase. The unmodified broth shows no increase until at least 23 hours.

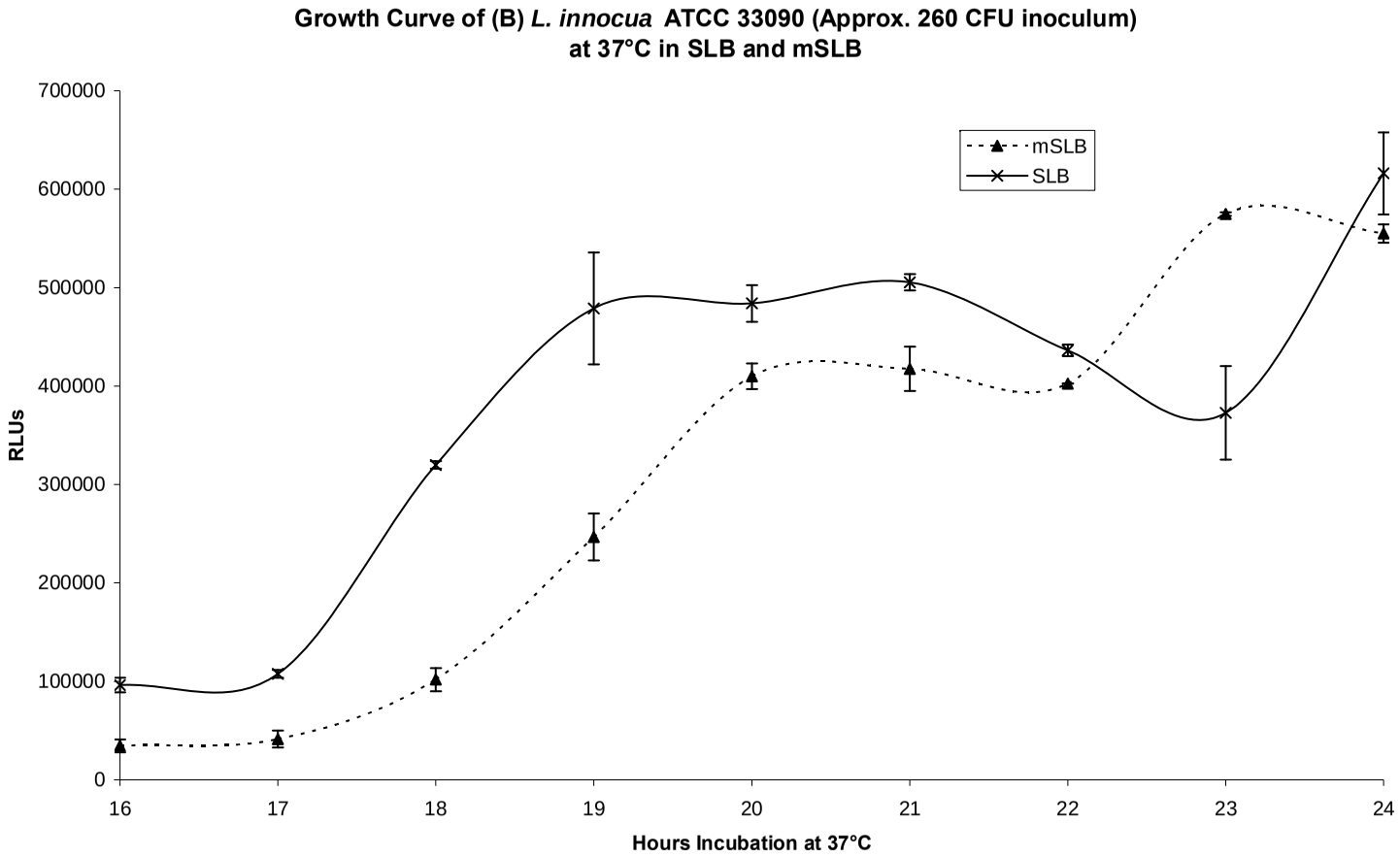



Figure 3.40: *L. innocua* shows exponential growth after 17 hours, and a plateaux at 19 hours. As with Figure 3.x, there is a dip in *Listeria* antigen CLISA detection between 21 and 23 hours, followed by another increase. The unmodified broth shows a faster increase but is otherwise similar.

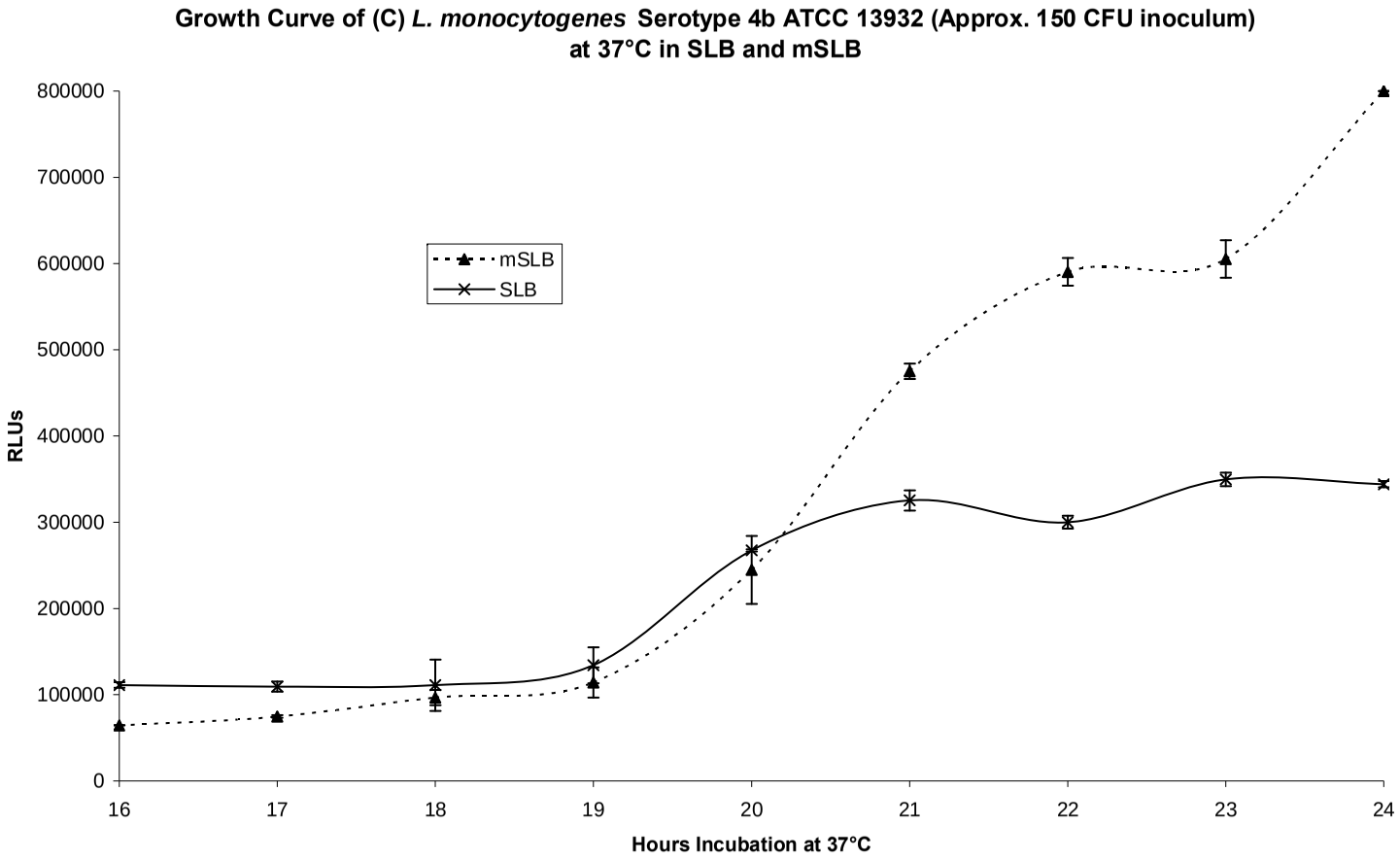



Figure 3.41: *L. monocytogenes* shows gradual increase, peaking at 21 hours. There is a dip in *Listeria* antigen CLISA detection at 22 hours. The reason for this is unknown. This was followed by another increase. The unmodified broth shows no increase until at least 23 hours.

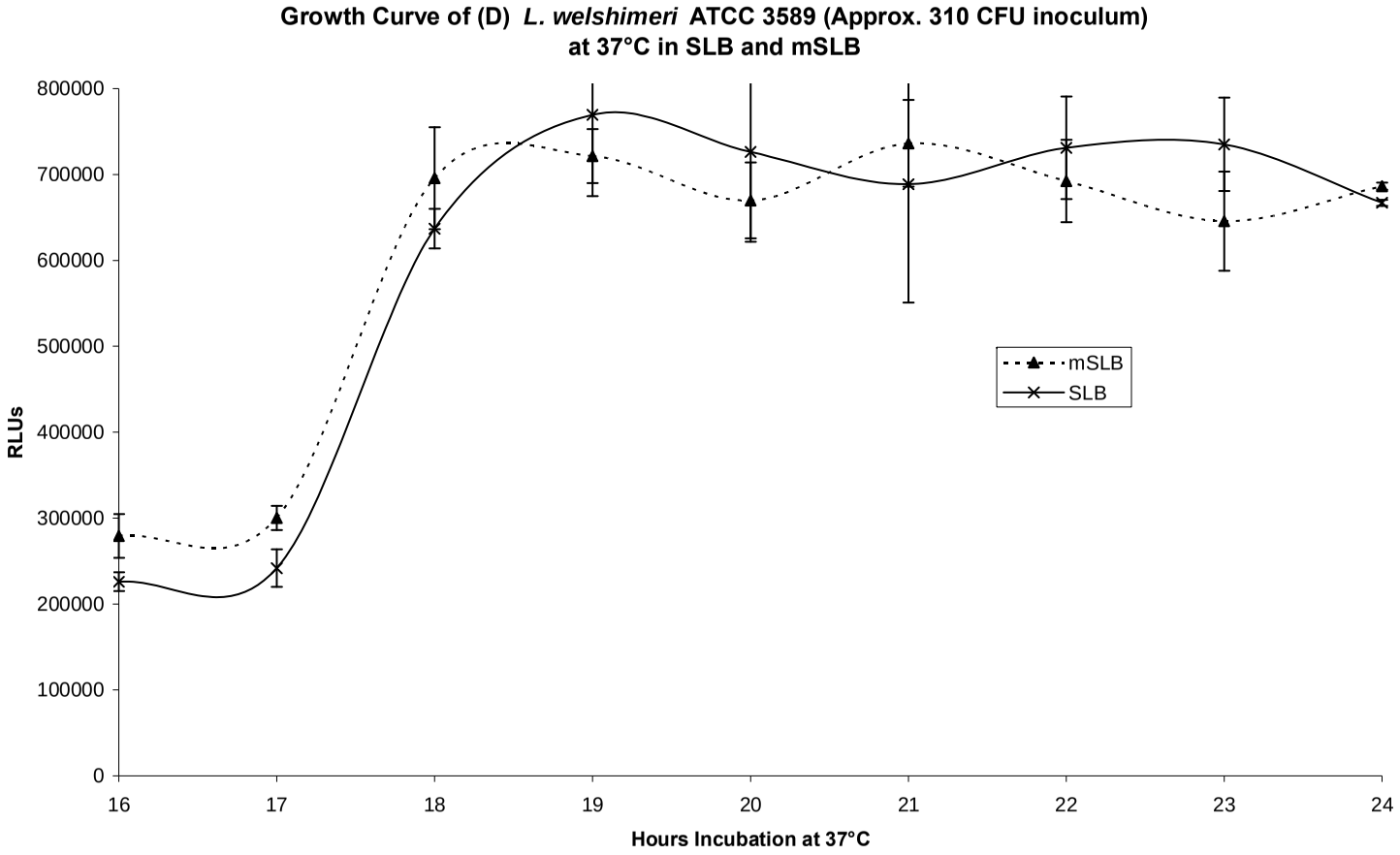



Figure 3.42: *L. welshimeri* was found to be one of the more rapidly growing strains in agar and the growth curve confirms that this is also true in broth. At 17 hours, growth becomes exponential until it flattens out an hour later. There is no significant difference ($P < 0.05$) between either broth.

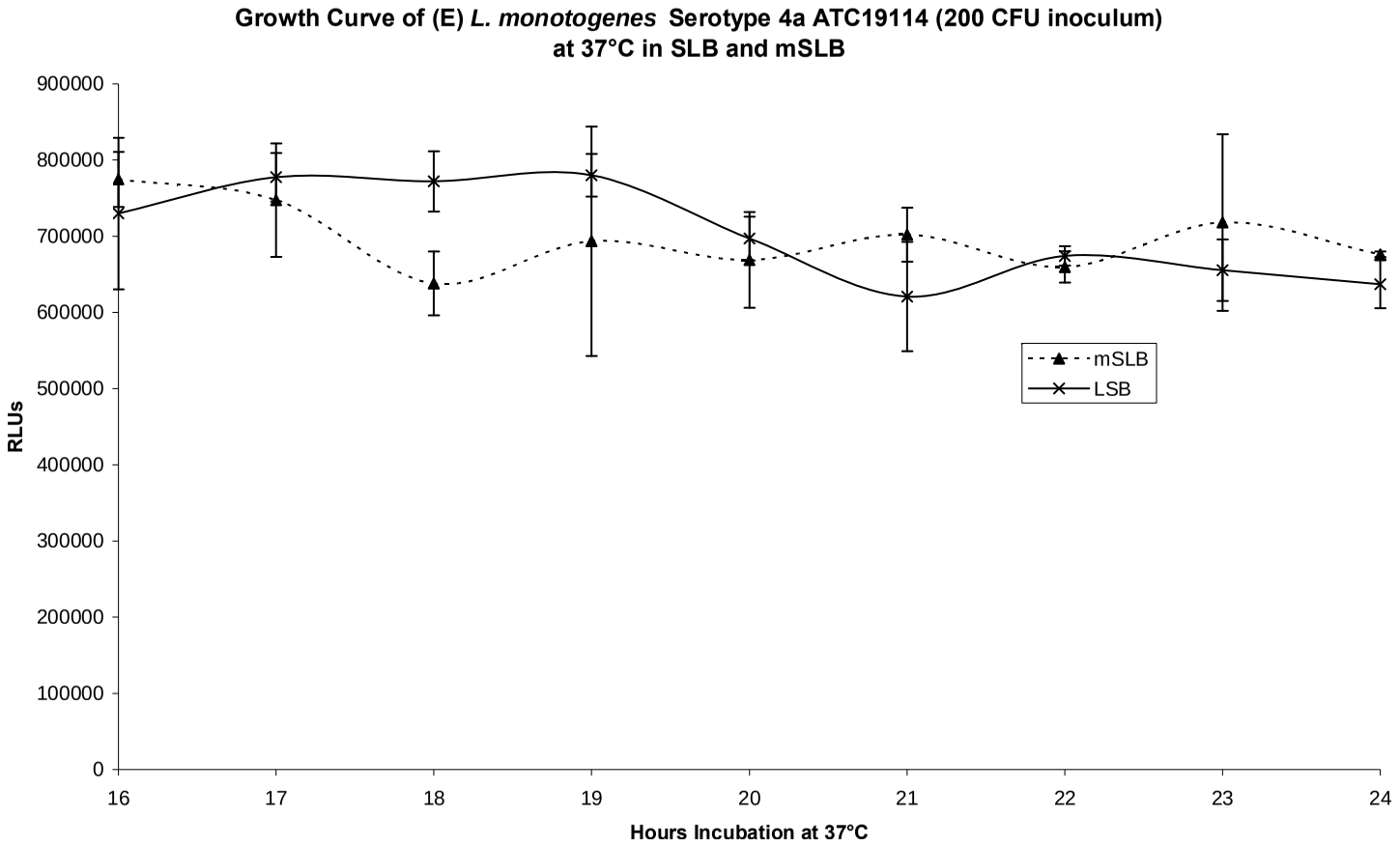



Figure 3.43: *L. monocytogenes* serotype 4a, which is not a pathogen (according to the supplier, LMG), reached a growth plateaux before the 16 hour mark regardless of broth. Non-virulent strains of *Listeria* evidently have growth advantages even at 37˚C. This may be due to their not wasting energy or resources on expressing virulence factors that do not assist their uncontested growth in-vitro.

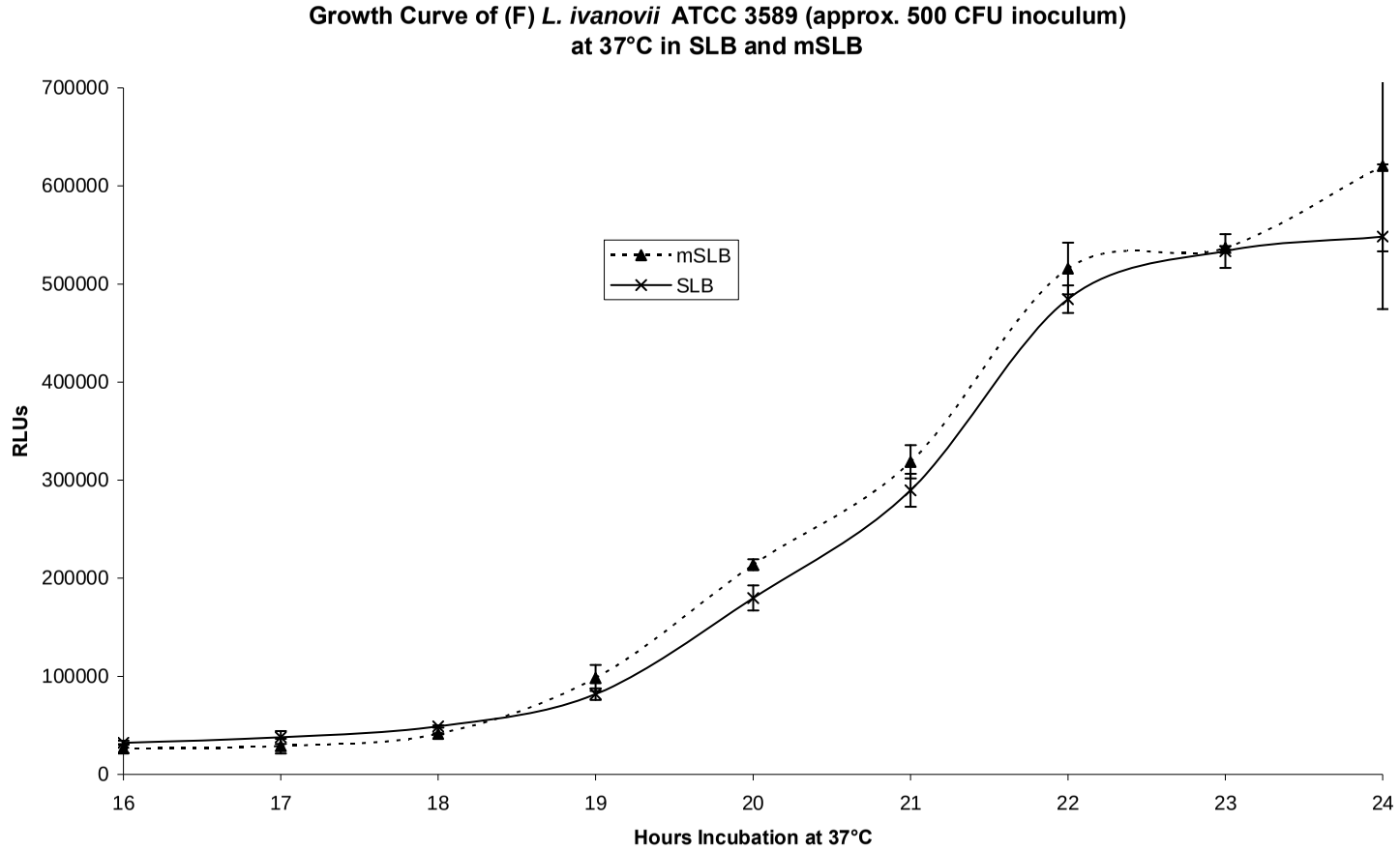



Figure 3.44: *L. ivanovii* was one of the more slowly growing strains on agar, and from its growth curve it can be seen that this extends also to growth in broth. CLISA antigen detection increases slowly until 19 hours, and then modestly until 21 hours where reaches a plateaux. There is little or no statistical difference in broths ($P < 0.05$). There is again a dip at 23 hours.

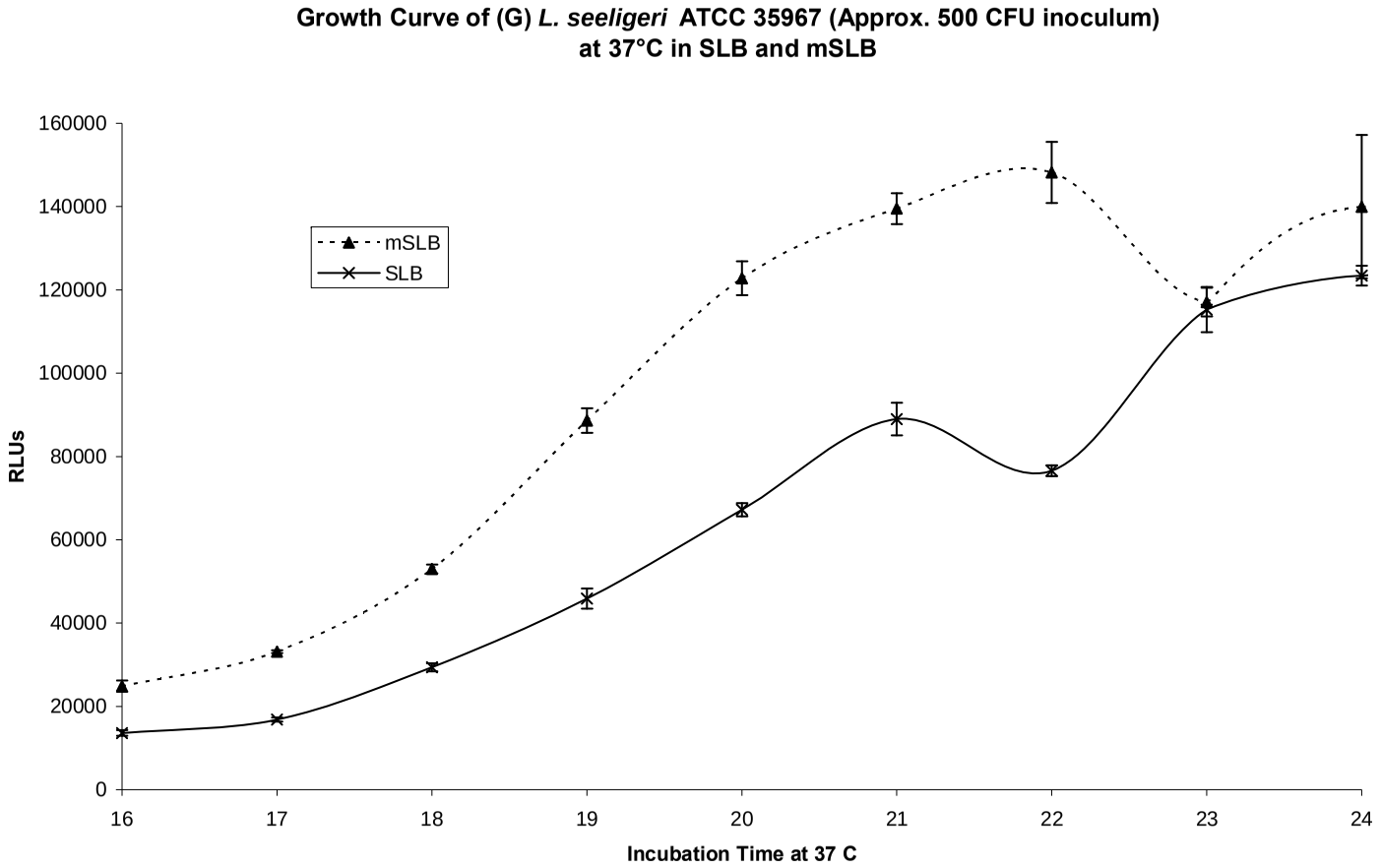



Figure 3.45: *L. seeligeri*, despite being poor on agar, grew relatively well in broth, with the modified broth having a clear advantage. As with previous growth curves, there is a dip at 22 hours for the unmodified broth, and at 23 hours for the modified broth.

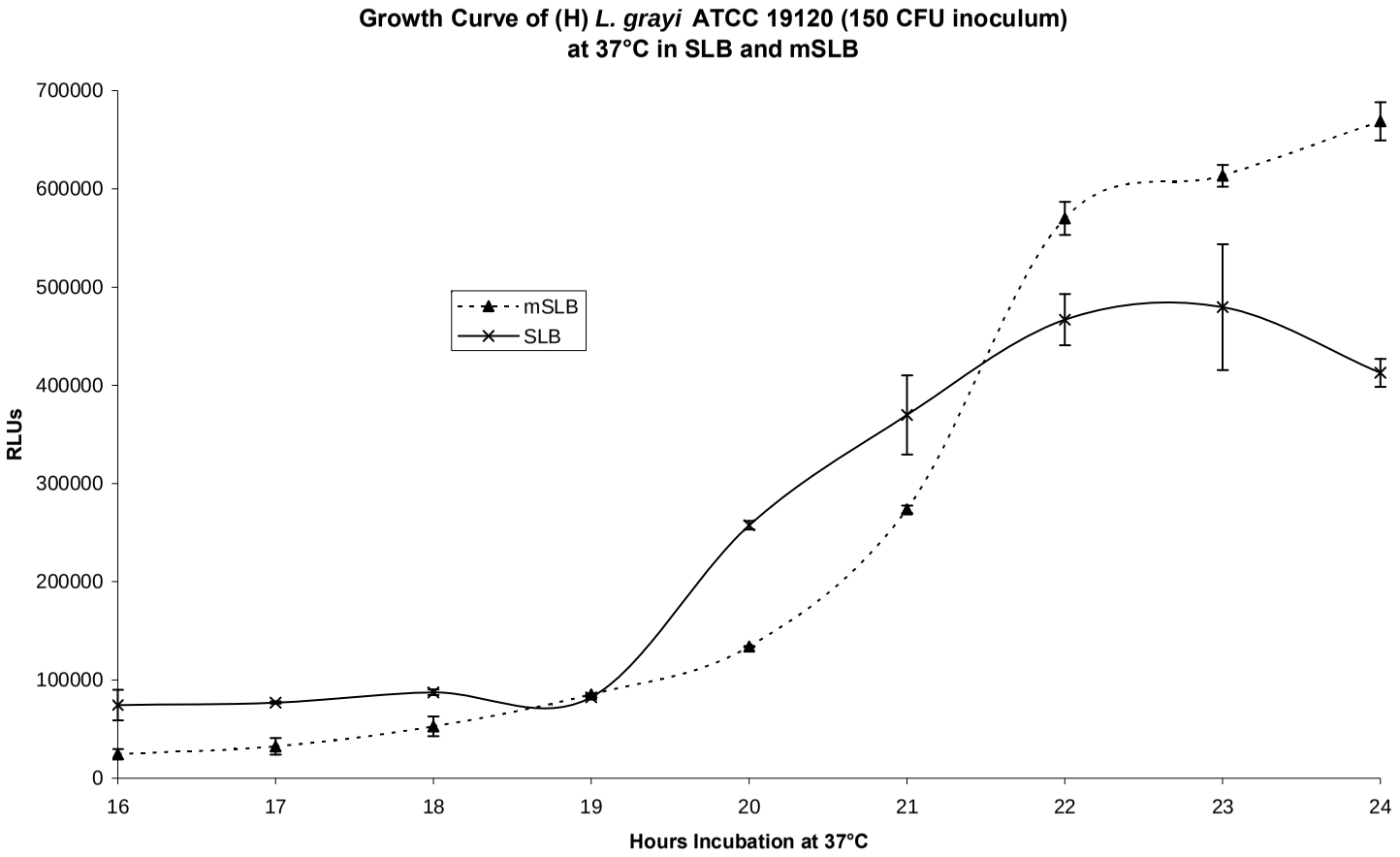



Figure 3.46: *L. grayi* grew very gradually until 19 hours unmodified broth before growing rapidly and peaking at 23 hours. In the modified broth, it took longer to reach a more rapid growth phase, and growth was greater after 22 hours. *Listeria grayi* may have some vulnerability to the antibiotics that other *Listeria* strains tested do not seem to have.

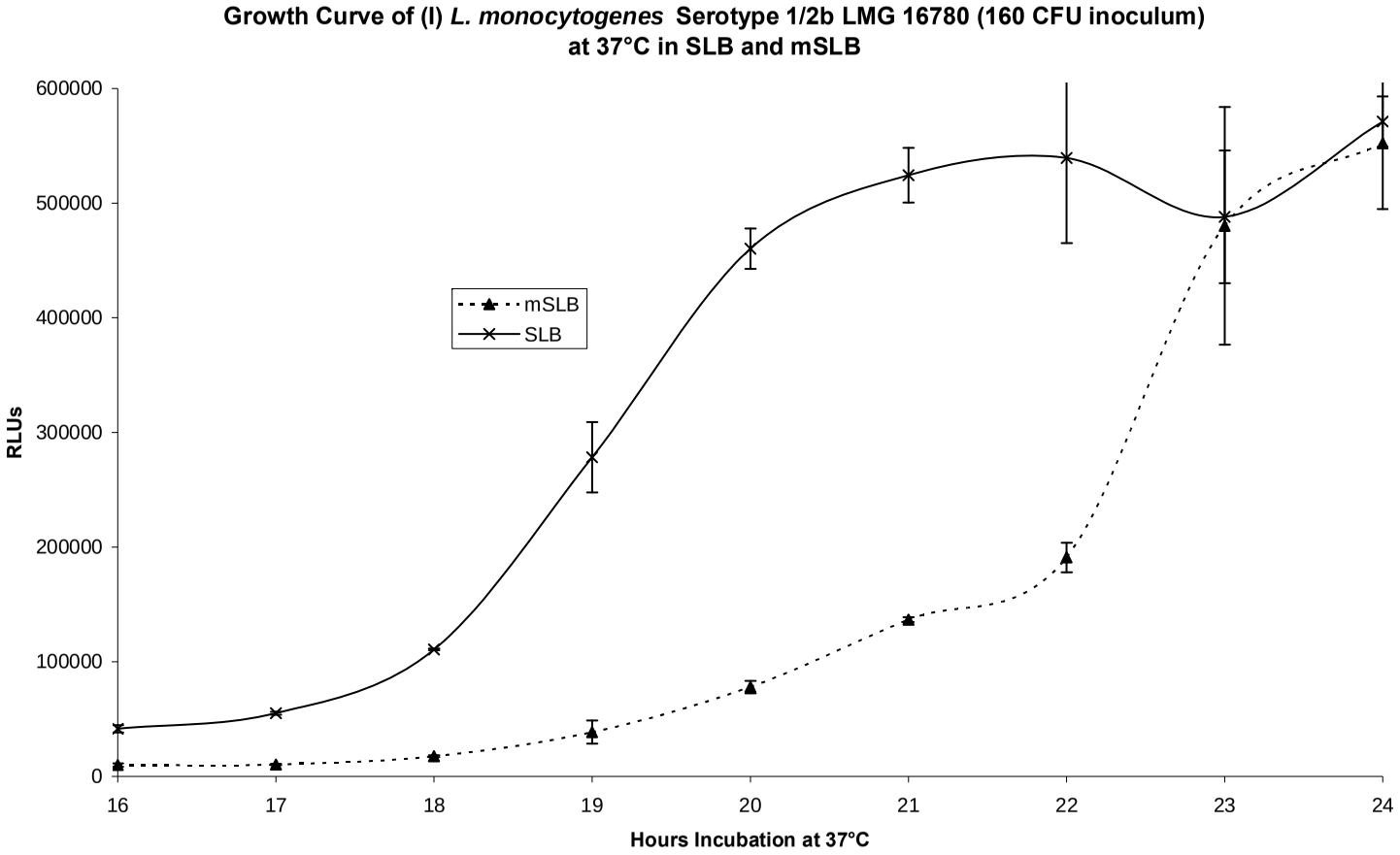



Figure 3.47: *L. monocytogenes* serotype 1/2b clearly did not grow as well in modified broth, with antigen detected by CLISA increasing very slowly until 22 hours. In stark contrast, the unmodified broth supported exponential growth after 18 hours, with a plateaux by 21 hours and a dip at 23 hours. This clearly illustrates the potential fallacy of assuming that all strains of *L. monocytogenes* will be similar, even those of the same serotype.

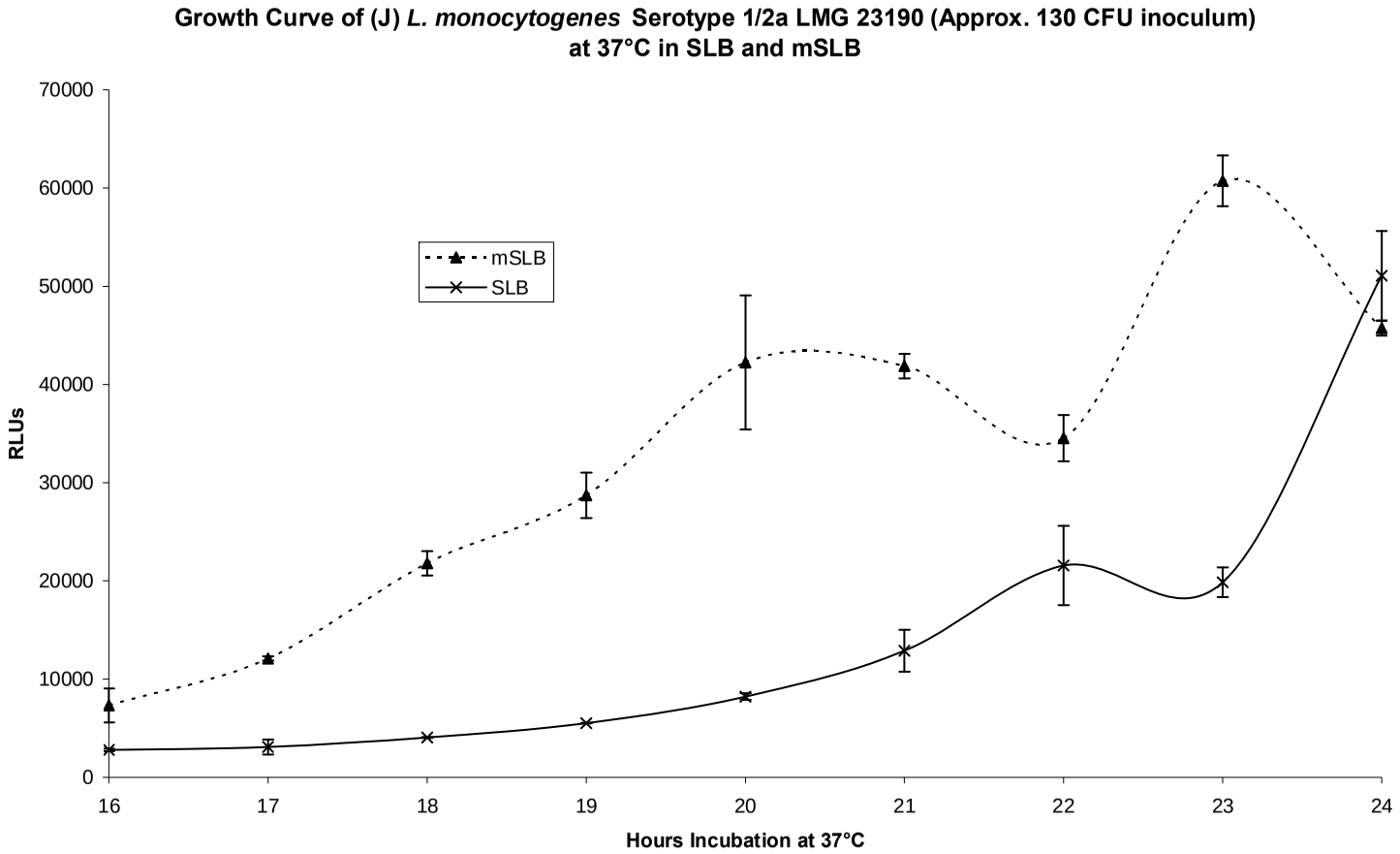



Figure 3.48: *L. monocytogenes* serovar 1/2a shows again how differently strains of the same species can respond. In this case, the modified broth was clearly better than the unmodified broth. The modified broth shows a more rapid increase in growth, and growth peaks one hour sooner.

# Chapter 4: Discussion

## 4.1 Production of monoclonal antibody against *Listeria* spp.

### 4.1.1 Success of hybridoma fusions

After four hybridoma fusions, there were eighteen selected hybridoma cell "well" mixtures, of which two were cloned: F2-4C4 and F4-IF2. F2-4C4, which was cloned was identified as a $IgG_1$ and mAb expressed detected 9 of the 10 *Listeria* strains, giving some hope that this immunisation method could produce a monoclonal capable of detecting all *Listeria*, although it completely fails to recognise *L. monocytogenes* serovar 1. This line unfortunately proved to be unstable: it would stop expressing antibody after a few generations, and had to be abandoned.

F2-5E5, although not cloned, also gives some credence to the possibility of producing a monoclonal that is specific only to antigen(s) found in all *L. monocytogenes* strains. Supernatant from these cells produced Ab that seemed to be specific to *L. monocytogenes* regardless of serotype, and weaker with the other *Listeria* species. It could form the basis of a specific anti-*L. monocytogenes* and make much of the work to find an antigen specific only to *L. monocytogenes* unnecessary. It may even be possible to use the antibody to discover the antigen and use it as a basis for further work.

The cells in well F2-4E10 displayed another broad anti-*Listeria* but failed to recognise *L. grayi*. Wells F2-5B4, F2-5E2 and F2-4D6 were less interesting because of patchy response to *Listeria* species.

The third fusion produced a great number of positive wells, but on further examination, the majority of these detected only a single strain of *Listeria* and are not documented in this thesis. Seven promising positives were isolated (see section 3.5.4) although none detected all strains and exhibited no discernable pattern of detection. None were cloned due to time constraints, but were preserved for future work.

The fourth and final fusion was afforded more scrutiny because of the quick success of cloning one well, F4-1F2. This clone was discovered to be an IgM mAb, and on discovery of this other positives supernatants were isotyped as well, which was not done in earlier screenings. It was discovered that most of the anti-L*isteria* response from this fusion was due to IgM as well.

The monoclonal F4-1F2-4C8 performed relatively poorly in terms of signal and reactivity compared to KPL BacTrace or sheep serum pAb. Even at a very high coating concentration, signal was relatively poor, especially for *L. innocua* and *L. seeligeri*; the clinically vital *L. monocytogenes* serovars 4b and 1/2a being detected poorly or not at all. The low overall signal may be in part due to the impurity of the capture antibody, but even so, it is clear that this antibody alone could not be expected to detect all *Listeria* regardless of the trace antibody employed. It is also worthy of note that KPL BacTrace high signal against for *K. zopfii* was been eliminated by using F4-1F2-4C8 as a capture, although not all of the detection of *Bacillus* had been removed.

### 4.1.2 Cloned IgM line and the value of IgM in assay formats

The presence of IgM-expressing originator B-cells in heavily immunised mice was certainly surprising, but even more so that they were almost the only anti-*Listeria* Ig component. In a mouse after three immunisations, the isotype maturation process should have passed to completion, and isotypes isolated should have been almost exclusively IgG. The mice had been immunised monthly for over a year. The reason for the almost complete ubiquity of IgM in fusion 4 positives was unknown, but may have been due to the age of the animals at sacrifice (approximately 18 months).

IgM is problematic as an antibody meant for industrial bulk production, and even as an antibody meant for small scale test assay. Its pentameric form proved sensitive to denaturisation with even slightly alkaline pH and therefore not easily labelled with NHS esters, so it is of limited use as detection/trace antibody in future work. From the results presented in this thesis it may be possible to use it as a capture antibody, but a

capture antibody needs to be of high purity otherwise the contaminating proteins will compete for limited surface binding space, and IgM is not easy or economic to purify to the necessary standard. It does not bind to common affinity matrices such as protein A, protein G or protein L[38].

Thiophilic absorption was used in an attempt to purify IgM from F4-IF2-4C8 monoclonal supernatant. This technique allows the isolation of all Ig, including IgM[39]. This unfortunately means that it also binds bovine Ig from FBS used in culture medium as well as mouse Ig from hybridoma cells. This bovine component could theoretically be removed by passaging through immobilised protein G. In previous work (not part of this study), T-Gel purified mAbs tended to have an appreciable contamination of bovine alpha-2-macroglobulin from the same source.

Although IgM was proven as a workable capture isotype, it was difficult to puriify to a grade to make their use in a capture ELISA or CLISA feasible. While F4-1F2-4C8 may be of some limited value as part of a mixture of antibodies, because of its difficulty of processing as an IgM, and because of its incomplete anti-*Listeria* strain profile, it is not suitable for a commercial assay.

With the successful cloning and culture of monoclonal F4-IF2-1C8, antibody it produced was screened against all *Listeria* and the most pressing problem organisms, namely *Kurthia* and *Bacillus*. Although activity of the original well was known, the monoclonal isolated many not have the full anti-*Listeria* activity of that cell mix as it may have contained more than one anti-*Listeria* mAb expressing cell clone.

In order to remove the possibility of IgM expressing cells being isolated and cloned at the expense of IgG expressing ones, gamma light-chain specific secondary antibody should be employed in screening from the outset. It would also be preferable not to keep mice for more than 8 months after immunisation.

### 4.1.3 Analysis of the choice of immunogen

While the technique of immunising mice with a boiled mix of lysed *Listeria* cells of a pool of strains proved successful in that it was able to produce antibodies to *Listeria*, no supernatant from any of the cell wells or clones were specific to all of the ten *Listeria* strains chosen for this study.

Is difficult to elucidate why one serotype of *Listeria* would lack an epitope that was present in the rest of the strains and even strains of the same species, especially as serotypical H antigens should have been destroyed by being boiled.

From these results, it may be feasible that, with enough screening, a monoclonal could be produced that will detect at least all of the ten selected strains of *Listeria*, although the odds are evidently not in favour and so screening runs would have to be upsized. It may be necessary to alter the immunogen, although it is not immediately clear what alterations would be most effective, except perhaps the removal of interfering flagella-expressing strains.

In general there is no discernable pattern or reason to why some *Listeria* strains would be detected by a monoclonal antibody and not others. This is obfuscated by the fact that most of the wells were never cloned, and may contain multiple mAbs as some certainly were prove to do. IgM has poor specificity but high avidity[40], and it may be that cell well supernatants with a greater range of detection may have had this ability because they had IgM as a major component.

There is also variability between mice anti-*Listeria* Ab titre in titre even in the same immunisation group. While there are trends (*L. monocytogenes* 4b seems to elicit poor response, possibly due to a more pronounced lack of flagella when grown at 37°C), there is no discernable pattern. Since they are cloned mice and all received identical immunogen this should not be the case. Though cloned, genetically identical mice can react differently if things like acetylation of DNA or their population of micro RNAs (which can alter gene expression) are different[41].

Little is known about the variety or nature of immunogenic epitopes may be present in *Listeria* that could present an antibody target. They could be assumed to exist in three major forms: proteins, carbohydrates, and lipids (and lipid variants). Carbohydrates and lipids in bacteria are composed mainly of repeating subunits and so are generally less immunogenic than protein, although they make up the majority of a bacterial cell dry mass[42]. If proteins involved in the pathogenicity of *Listeria* (virulence factors) were a prime epitope, then one could assume that only the virulent strains would be detected, but there were no wells that showed this behaviour apart from perhaps F2-5E5 and this showed slight cross-reactivity towards non-pathogens as well and had not been cloned.

The only *Listeria* antigen that is known with any degree of certainty is that, or those, associated with flagella. The Bioline assay proved that flagellar antigen was still present even in boiled *Listeria* that had been cultured at 37°C. Only the non-pathogenic strains were able to express these antigens when grown at physiological temperatures. This fits well with current theories on *Listeria* virulence, as flagella would provoke a potentially greater immune response in mammals via activation of a specific innate immune receptor, Toll-like receptor 5 (TLR5), that recognises the main protein in flagella, flagellin[43], thus immediately compromising non-virulent strains *in vivo*.

The presence of flagellar antigen anywhere in samples boiled for twenty minutes poses another question. The Bioline kit claims to detect flagellar antigens, although it also obviously detects them in bacteria boiled for 20 minutes. Anti-*Listeria* latex agglutination testing showed negative response with heat treated *Listeria*. However. heat treatment destroys the structural form of flagella but only slightly affects the immunogenicity of their components[44]. It is reasonable to assume that the target antigen detected by the proprietary monoclonal antibodies used as detection and capture by the kit must be a heat stable flagella-associated determinant.

Interestingly, even at 37°C culture, several strains of *Listeria – L. innocua*, *L. welshimeri, L. monocytogenes* serovar 4a and *L. grayi* – are detectable by the Bioline kit. From this it can be inferred that they still produce flagellar proteins even when grown at 37°C.

According to their NCTC catalogue entries, these strains are non-pathogenic to either humans or animals. Non-virulent strains of *Listeria* may be missing the mechanisms required to thermoregulate their loss of flagella. It is likely that the loss of highly immunogenic flagellar protein is only beneficial to the organism as a pathogen and not as a saprophyte. Note that the presence of these antigens does not necessarily mean that operational flagellae are fully expressed and the cells are motile at 37°C. This was not tested.

It is recommended that in further work, only *Listeria* strains that do not express flagella at 37°C be used for immunisations to avoid possible bias in response toward these immunogenic cell components.

### 4.1.4 Future work on monoclonal antibody production against *Listeria*

The immunisation protocol was simplistic and it is suggested changes could be made in the protocols to improve the chance of a useful mAb being produced. First is simply wider screening; with the assumption that a single epitope exists that is common to all *Listeria* species and strains, it becomes ever more likely to find an mAb expressed toward it in a fusion with greater number of cells and screening wells. The use of an anti-gamma anti-mouse IgG secondary Ab for screening is also advised. At the start of this work, the probability of IgM mAbs reaching the hybridoma fusion process was considered low, and the suitability of an IgM was unknown. It is now known that IgM are not suitable, and their presence on screening plates only lowers the chances of finding a suitable IgG expressing cell line.

One possibility to improve the titre of Ab specific to *Listeria* cell wall components is the removal of strains that express flagellar antigens. As described in section 4.1.3, these proteins and components may produce a greater immune response against them than other cell components, and hence bias the mAb produced toward detecting them. Removing this bias should again increase the chance of producing a mAb useful for detection of all *Listeria* grown at 37°C.

Future work on the production of anti-*Listeria* monoclonals could also look into the possibility of determining what the most common epitopes are in *Listeria*, especially for mAb with a broad range of detection across the strains. A possible way of doing this would be to immobilise mAb produced in fusion (for example on cyanogen bromide activated sepharose) and use it to isolate the corresponding antigen. If protein, this could then be run on SDS-PAGE and even sequenced, for example by Edman degradation[45]. If identification is practically impossible, the purified antigen could be used to re-immunise animals directly and increase the possibility of a mAb with broader specificity by eliminating interfering factors from the *Listeria* cells.

## 4.2 Production of polyclonal antibody against *Listeria* spp.

### 4.2.1 Immunisation of sheep with *Listeria*

Immunisation of sheep with the same *Listeria* cell components produced an antibody response as expected. The development of polyclonal antibodies initially seemed more promising than the monoclonal development, to the point that it seemed almost that it would make it irrelevant in terms of selection for an assay. Although antiserum from sheep immunised with *Listeria* had high-cross reactivity when used directly, when it was immunopurified with immobilised *Listeria* antigens, any observable cross-reactivity disappeared towards all bacteria species tested, including those common in foodstuffs. This confirms the presence of epitopes unique to *Listeria* and found on all *Listeria*. The identity or nature of these epitopes are unknown.

### 4.2.2 Immunopurification of sheep pAb

The phenomenon of *Listeria* antigen binding to the sepharose support was not expected to work when attempted and its mechanism is not clear. Sepharose activated with cyanogen bromide has active cyanate esters or imidocarbonate groups. These react readily with primary amines, mainly those found in surface lysine residues in protein[46]. Protein immobilisation on cyanogen bromide activated polysaccharides is well documented, but carbohydrate immobilisation is far less well explored. Cell wall

carbohydrates (in gram positive bacteria mainly peptidoglycans and teichoic acids[47]) may have proteins or available primary amines imbedded within sections of them that may in theory covalently bind to the cyanate esters, but this again is not documented in any literature.

Presumably the commercial KPL BacTrace polyclonal antibody is immunopurified in a similar fashion as used in this study, but probably with a more refined technique. There is more emphasis on affinity rather than specificity: KPL could afford not to be concerned with cross-reactivity with *Kurthia* or *Bacillus* because these were not problem organisms in the 48 hour Fraser and Half Fraser broths used in the food assays the antibody was purposed for, and so the KPL BacTrace antibody produces higher signal, and therefore greater sensitivity. In correspondence, KPL claimed to have purified the antibody using "positive" and "negative" absorption stages. Presumably the positive absorption works like the immunopurification undertaken in this study. What antigens KPL immobilised or what chemistry or matrix material they did so with has not been published. It did not prove necessary to absorb out any unwanted cross-reactivity to non-*Listeria* bacteria from the immunopurified mAb as none was observed among the species tested, including those that were able to grow in unmodified SLB.

It should also be noted that the specificity of immunopurified sheep pAb is greater with *L. welshimeri* and *L. monocytogenes* serovar 4a (Section 3.4.4). It is not unreasonable to suggest that antibodies toward highly immunogenic cell components like flagellae make up a large part of the antibody response even against cells grown at 37°C. To avoid this, and potentially increase the titre to non-flagellar cell components, these strains and any other strains which express flagella at 37°C could be omitted when pooling antigen for immobilisation to sepharose resin in future work.

From the results of the immunopurification of serum from the immunised sheep, it appears that only a small minority of IgG from an immunised animal is specific to the immunogen, approximately 0.5 – 2%. This study was able to purify 45 µg of anti-*Listeria* pAb per 1 ml of serum. Because it was not purified by protein A or G, it is certainly a mixture of Ig isotypes including IgM, with the actual IgG content being an unknown fraction of this.

This relative scarcity of specific IgG is not a problem as serum is a virtually inexhaustible resource, although in production the relative expense of the immunopurification technique in general may raise issues. Large scale purification of antibody may use support matrices and materials resistant to high throughput pressure such as glass beads, while there were no CNBr-activated glass matrices available on the market as of the time of this thesis. There may also be limited longevity of the bound antigen compared to techniques such as protein A sepharose, but this has not been confirmed.

### 4.2.3 Non-specific binding of sheep pAb to immobilised sheep pAb

The very high background signal when the pAb immunopurifed in this study was used on both sides of a sandwich assay prohibited its use in sandwich assay. The explanation for this phenomenon may be an autoantibody response known as rheumatoid factor. Rheumatoid factor is usually defined as a population of serum antibody specific to specific to the Fc region of IgG of the same species[48]. Although usually IgM, it can in fact be of any isotype[49]. As a clinical manifestation, the presence of rheumatoid factor is usually associated with a past infection with gram positive bacteria through an unknown mechanism[50].

Obviously autoantibody has not bound to IgG in solution, as it would have aggregated and precipitated as a high molecular weight mass. When antibody is bound to the activated polystyrene surface of a microtitre however, it may possibly have provided a conformational change in the IgG that made it a target of autoantibody in purified pAb, resulting in a very high background as detection antibody bound to the coating antibody. Immunoaffinity binding to an immobilised antigen should only leave Ab that is bound to the antigen to be eluted. In this case, when the Ab has bound to the immobilised antigen, it has again become a target of autoantibody that has bound to it and been immobilised on the sepharose by binding to anti-*Listeria* pAb, only to be eluted along with it when the pH was acidified.

A possible method to remove this unwanted binding component in the pAb may be to purify the IgG component of serum through a typical affinity chromatography method such as immobilised protein A; this would assume that the greater part of the autoantibody component was IgM. Protein A binds IgG via the Fc region[51], so in theory this epitope would be hidden from the majority of autoantibody and therefore it should not bind to the IgG; and as IgM it would not be bound by protein A, and should be washed out with the other serum proteins. This is an easy solution in regards to large scale production, as protein A is relatively cheap and re-usable many times. As an autoantibody removal method it would however fail if the greater part of the autoantibody was IgG, as the protein A would also bind the autoantibody.

Another more effective means of removing all autoantibody may be passaging the immunopurified sheep antibody through sheep polyclonal IgG bound to CNBr-activated sepharose in the same way the *Listeria* antigen was. Rheumatoid factor should bind and all other pAb be allowed to pass through. This would however necessitate another dedicated step with a softer sepharose matrix and thereby complicate mass production; and because of the potentially limited binding capacity of this method it may not be able to remove all of the autoantibody from larger batches of serum in one run.

## 4.3 Selective *Listeria* Broth development

### 4.3.1 Selective *Listeria* Broth unmodified with food

Selective *Listeria* Broth, SLB, was originally intended to be developed and altered to complement the strengths and weaknesses of antibodies produced in this study. The first test of SLB revealed problems with other microflora that needed to be solved before the broth could be used with foodstuffs regardless of antibody or even detection method. This was the complete suppression of *Listeria* growth by other gram-positive bacteria and the first challenge that had to be overcome.

Before this study, Selective *Listeria* Broth (SLB) was known to support the growth of *Listeria*, and to be suppressive of other non-specified bacteria. Its effectiveness of a

wider range of organisms had never been investigated. As selective components unmodified SLB has lithium chloride and nalidixic acid. Lithium chloride gives medium selectivity by inhibiting salt-intolerant organisms, mainly gram negative bacteria: *Listeria* is not affected due to its high salt tolerance (described in section 1.2). Nalidixic acid is a synthetic quinolone antimicrobial compound that acts in a bacteriostatic rather than a bacteriocidal manner; as a quinolone, its method of action is the inhibition of bacterial DNA gyrase[52]. *Listeria* is highly resistant to nalidixic acid, possibly due to amino acid substitutions in its DNA gyrase[53].

### 4.3.2 Modification of SLB for use with foodstuffs with high bacterial load

The SLB in its original form was not sufficient for inhibiting *Listeria*-suppressing microbes at the kind of numbers and diversity found in most kinds of uncooked food, particularly those with high numbers of endogenous bacteria such as raw meat or dairy produce. SLB failed completely to support the growth of detectable numbers of *Listeria* on haddock fillet and beef mince. This was proven by the negative results on chromogenic agar from SLB broth to be a failure of the *Listeria* to grow in the combination of this broth and food, rather than a failure to detect its presence by CLISA. Given the high aerobic plate counts of both foods, it seemed very likely that the presence of an organism or organisms inhibitory to *Listeria* was responsible for its total lack of proliferation. On isolation of colonies when streaked out onto agar, these were observed to be mainly enterococci, Pseudomonads and *Kurthia* spp. in raw meats.

A study of antibiotic susceptibility of *Listeria* spp. was used as a foundation for the choice of additional selectives[54]. Although the study was carried out in a proprietary, minimal culture medium, the paper proved very useful for identifying potential antibiotic choices and a very approximate starting point for their concentrations in SLB.

Nitrofurantoin, a nitrofuran antibiotic, is activated by bacterial flavoproteins (nitrofuran reductase) to active reduced reactive intermediates that modulate and damage ribosomal proteins or other macromolecules, such as DNA. This inhibits

DNA, RNA, protein, and cell wall synthesis which causes cell death[55]. Nitrofurantoin is stated in literature as effective against enterococci, staphylococci, streptococci, corneybacteria, and many strains of *Escherichia coli*[56]. In food testing this effectiveness was confirmed in SLB as 12 mg $L^{-1}$ nitrofurantoin all but eliminated any cocci growth in broth. However it made little or no difference on any other organism. Higher doses were not investigated as these affected *Listeria* growth.

Cefsulodin proved effective against Pseudomonads. Cefsulodin is a third generation cephalosporin antibiotic that is almost exclusively active only against *Pseudomonas* and *Aeromonas*[57]. This narrow activity meant that it could be used at relatively high concentrations without affecting *Listeria* growth if the need arose. However the broader activity of ceftazidime, its effectiveness against Pseudomonads even at low dose, and with the economics of this smaller dose made it a better choice. Ceftazidime is also a third generation cephalosporin. It was investigated because of its common use in *Listeria* selective broths and agars, including OCLA.

The addition of 2 mg $L^{-1}$ ceftazidime to complement nitrofurantoin was found to retard the growth of almost all of the microbes inhibitory in *Listeria*, at least those found in the foods tested. This was with the exception of dairy products. In addition, this modification did not affect the rapidity of growth of *Listeria*; it seems to have *increased* growth through some unknown mechanism. SLB supplemented with ceftazidime and nitrofurantoin was an effective selectice broth for all foods except dairy produce.

It may have been useful to test more food samples and more food types with which the final commercial assay is intended to screen. However, these could not be tested due to time constraints. It would also have been ideal to have been able to assay reference samples with a known quantity of the target antigen using this broth and assay; however, reference samples of food spiked with a known number of *Listeria* CFUs were not available at the time of this study,

### 4.3.3 Modification of SLB for use with dairy products and dried milk powders

Ceftazidime and nitrofurantoin were not sufficiently active against the microflora of dairy products when cultured for 20 hours at 37°C. Performance was erratic and differed even between different samples of the same food. CLISA of the cultured broth would sometimes fail to detect *Listeria*. When the failed samples were streaked out onto plates, they normally would show the presence of furry *Bacillus* colonies or blister-like *B. licheniformus* colonies. Samples where *Listeria* was detected by CLISA would produce *Listeria* colonies as expected, with few other types of colony.

Most markedly difficult to culture *Listeria* in with broth was infant formula milk, which consistently failed to support the growth of *Listeria* even with high inoculum. As a concentrated, pasteurised powder (not ultra heat treated), infant formula milk powder had a very high number of gram positive bacterial spores, mainly *Bacillus* and close relatives like *Brevibacillus*. Upon adding 25 g of dry powder to SLB at 37°C, these were capable of very rapid recovery and growth, easily out-competing any *Listeria* inoculum and wholly suppressing its growth. Many *Bacillae* are capable of expressing antibiotics and bacteriocins[58] which may have been responsible for this suppression. Their growth was also exceptionally rapid. When streaked on SLB, *Bacillae* would be observed to form visible furry colonies in mere hours at 37°C.

It is suspected that, along with the expression of the more immunogenic structures of flagella, *Bacillus* is one of the major reasons why *Listeria* is normally cultured at 30°C in food test kits such as that of Bioline. According to the instuctions supplied with the kit, the broth in the Bioline kit, RELM, contains the same basic selective agents as base SLB (nalidixate, lithium chloride) along with only acriflavine as an additional selective. Acriflavine was totally ineffective in the tests in this study. The optimum growth rate temperature for most common *Bacillus* spp. are above 35°C[59]. It is likely that the lower temperature changes either the growth characteristics of this wide genus or its expression of compounds deleterious to *Listeria*.

Cefotetan, a second generation cephalosporin, is a component of Oxford agar. When *Bacillae* were noticed to fail to form colonies or to grow poorly on Oxford agar, cefotetan was investigated as a possible supplement to modified SLB. When added to

mSLB it could control heavy populations of *Bacillae* to the point where *Listeria* could grow. Limited testing of cefotetan added to mSLB hinted at a greater suppression of *Listeria* growth than mSLB alone, although this was not quantified. This and the added costs of cefotetan probably mean that cefotetan would be recommended as a supplement only for foodstuffs such as dairy products, as it would reduce the sensitivity of the assay in food materials that do not require it.

### 4.3.4 Limitations of the ISO method found in this study

While the ISO method is considered the official confirmatory test in food testing laboratories, some limitations were also discovered in the course of this work. While the use of Fraser and Half Fraser broths excluded almost all competing microflora, both ALOA and Rapid L mono agars still necessitated the use of agglutination assay to positively identify apparent positives as *Listeria*. Unknown coryneform or *Bacillus* spp. were capable of not only of passing the Fraser broth steps but growing rapidly on ALOA when streaked. They looked like *Listeria* both in terms of colony morphology but also the chromogenic reaction caused by ß-glucosidase. *Listeria* plates in an incubator produced a faint, bread-like odour due to acetoin production; the unknown bacteria produced a stronger, unpleasant and penetrating cheese-like smell. However, *Listeria* could be readily be discriminated by latex agglutination as long as it was cultured at 30°C for at least 24 hours.

Several obvious *Bacillae* were also capable of growing on ALOA and Rapid L Mono plates and of lecithinase production, but their colony morphologies were very different. *B. circulans*, if present, and the "coryneform" (believed to be *Kurthia* spp.) as found on several plates on the ISO method, were often indistinguishable from *L. monocytogenes*. Small white, round colonies on Rapid L mono could be a (non *L. monocytogenes) Listeria* spp. but could equally be *Micrococcus* spp. This was ubiquitous on Rapid L mono, even from Fraser broth. The coverage of eventualities even on both types of plates was not sufficient to dispense with the expensive and time-consuming agglutination step without significant uncertainty. Oxford agar proved difficult to read as a small cluster of *Bacillus* on the plate would often turn the whole plate black. Other types of chromogenic agar on the market would result in different

issues but the same outcome: colonies that could not be absolutely defined as *Listeria* without subsequent tests (such as latex agglutination), time, and expense. Clearly there is potential to replace the whole ISO method even as a baseline confirmatory test, beyond the scope of development of an ELISA or CLISA assay.

### 4.3.5 *Listeria* growth in SLB and modified SLB

Visual inspection of the growth plots of *Listeria* strains cultured in SLB and SLB modified with nitrofurantoin and ceftazidime hints at there being no measurable *Listeria* numbers until around 16 hours, at least for most strains, with *L. welshimeri* and *L. monocytogenes* serotype 4a being the exceptions. There seems often to be a small "dip" or decline in the CLISA output between 18 and 23 hours, depending on the strain and number of original inoculum. It is impossible to tell whether this is a decline in cell numbers or a change in the concentration of antigen, change in expression of the antigen, or some other such mechanism. It is however a real phenomenon as it occurs on almost every growth curve and the time of its appearance can be visually associated with the initial rapidity of growth.

*L. monocytogenes* serotype 4a is so rapid-growing and the KPL antibody has such a high affinity for this strain that it had already peaked by the 16 hour mark and a growth curve was not possible. Clearly its growth in mSLB is not affected in any significant way. *Listeria welshimeri* also reaches peak after about 18 hours. For the others, this small "dip" in signal is delayed for 1-2 hours in the original, un-modified broth. In all cases though, all *Listeria* strains are detectable after 20 hours in both broths, although they generally peak between 21 and 24 hours. Only three strains of *Listeria* seemed to grow more poorly in mSLB: most notably *L. monocytogenes* serovar 1/2b, as well as *L. grayi* and *L. innocua* to a lesser extent. All are still detectable by the vital 20 hour mark.

*Listeria ivanovii* does not seem to have been affected by the modifications, and grows at the same rate as in the original broth. This is particularly important as *L. ivanovii* is the clearly the species and strain of those tested that grows most slowly in SLB and may have been problematic to detect from a small inoculum after 20 hours even if inhibition would have been slight.

The growth curves show that the modified broth not only is capable of supporting the rapid growth of *Listeria*, it is actually better than the original broth in most cases, despite the addition of potentially inhibitory antibiotics. The reason for this is not known.

## Chapter 5 Summary

This study was undertaken to develop an assay for the detection of *Listeria* spp. in food samples after 20 hours of culturing in a single broth at 37°C. Central to this was the development of monoclonal antibody lines against *Listeria*. Secondary tasks were the production of polyclonal antibodies against *Listeria* as an assay partner to the mAbs, and the development of a selective broth that could support the growth a single viable CFU of any *Listeria* spp. in food up to detectable levels after 20 hours, while suppressing the growth of any interfering organisms.

This study was partially successful in raising monoclonal antibodies against *Listeria* and *Listeria monocytogenes* alone, when the bacteria are cultured at 37°C. After four hybridoma fusions, cells from many wells proved positive, but two were cloned. One clone was unstable, but detected 9 out of 10 of the *Listeria* strains. None of the positive cell wells in all screening plates was able to detect all ten *Listeria* strains at once.

Sheep pAb isolated by immunopurification proved to be easier to produce and very specific. pAb purified by this method detected all ten *Listeria* strains, and showed very little or no binding to non-*Listeria* bacteria. However when immunopurified sheep pAb was used both as capture and detection Ab it exhibited very high levels of non-specific binding. This is believed to be due to the presence of autoantibodies in the sheep serum, although this was not proven.

A broth and a secondary broth supplement were developed that made it possible to culture a very small number of *Listeria* in all tested foods to the point that they could be detected by ELISA or CLISA in a complete 21 hour assay. Selective *Listeria* Broth (SLB) originally intended for this role proved insufficient in that it allowed the proliferation of many other bacteria other than *Listeria*. Addition of 12 mg $l^{-1}$ nitrofurantoin and 2 mg $l^{-1}$ of ceftazidime heavily suppressed most of these organisms and not reduce the rate of growth of the ten *Listeria* strains. The addition of a further selective component to the broth, 7 mg $l^{-1}$ cefotetan, proved necessary for dairy foods but not recommended for other types of food as it was slightly suppressive to *Listeria* spp..

Overall, production of a mAb to *Listeria* grown at 37°C may be possible by scaling up screening and adjusting *Listeria* strains used to immunise, but it may not be necessary if the non-specific binding of immunopurified pAb can be overcome. With the supplementation of Selective *Listeria* Broth, the full assay as stated in the original aim is already possible without a monoclonal antibody, although the commercial KPL antibody will be required as a Ab partner.

## References


[1] Murray EGD, Webb RA, Swann MBR (1926). A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *The Journal of Pathology and Bacteriology* **29**(4)**:** 407-439.

[2] Patel JR, Hwang CA, Beuchat LR, Doyle MP, Brackett RE (1995). Comparison of Oxygen Scavengers for Their Ability to Enhance Resuscitation of Heat-injured *Listeria monocytogenes*. *Journal of Food Protection* **58**(3)**:** 244-250.

[3] Portnoy DA, Auerbuch V, Glomski IJ (2002). The cell biology of *Listeria monocytogene*s infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *The Journal of cell biology* **158**(3)**:** 409-414.

[4] Purkrtová S., T. H., Pilchová T., Demnerová K., Pazlarová J. (2010). Resistance of *Listeria monocytogenes* biofilms to disinfectants. *Czech Journal of Food Science* **28**: 326-332.

[5] Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2003). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of medical microbiology* **52**(Pt 12)**:** 1065-1070.

[6] Abrishami SH, Tall BD, Bruursema TJ, Epstein PS, Shah DB (1994). Bacterial adherence and viability on cutting board surfaces. *Journal of food safety.* **14**(2)**:** 153-172.

[7] Wei QK, Fang TJ, Chen WC (2001). Development and validation of growth model for *Yersinia enterocolitica* in cooked chicken meats packaged under various atmosphere packaging and stored at different temperatures. *Journal of Food Protection* **64**(7)**:** 987-993.

[8] Division of Foodborne, Bacterial and Mycotic Diseases (DFBMD). CDC. April 4 2008.

[9] Portnoy DA, Auerbuch V, Glomski IJ (2002). The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *The Journal of cell biology* **158**(3)**:** 409-414.

[10] Guillet C, Join-Lambert O, Le Monnier A, Leclercq A, Mechaï F, Mamzer-Bruneel MF*, et al.* (2010). Human listeriosis caused by *Listeria ivanovii*. *Emerging infectious diseases* **16**(1)**:** 136-138.

[11] Perrin M, Bemer M, Delamare C (2003). Fatal case of *Listeria innocua* bacteremia. *Journal of clinical microbiology* **41**(11)**:** 5308-5309.

[12] Todeschini G, Friso S, Lombardi S, Casaril M, Fontana R, Corrocher R (1998). A case of *Listeria murray/grayi* bacteremia in a patient with advanced Hodgkin's disease. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **17**(11)**:** 808-810.

[13] "*Listeria monocytogenes*." Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. U.S. Food and Drug Administration. 4 April 2008.

[14] Farber JM, Peterkin PI (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological reviews* **55**(3)**:** 476-511.

[15] Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins - Second Edition, Food and Drug Administration, p.13.

[16] Antal EA, Hogasen HR, Sandvik L, Maehlen J (2007). Listeriosis in Norway 1977-2003. *Scandinavian journal of infectious diseases* **39**(5)**:** 398-404.

[17] Kendall P, Medeiros LC, Hillers V, Chen G, DiMascola S (2003). Food handling behaviors of special importance for pregnant women, infants and young children, the

elderly, and immune-compromised people. *Journal of the American Dietetic Association* **103**(12)**:** 1646-1649.

[18] Allerberger F, Wagner M (2010). Listeriosis: a resurgent foodborne infection. *Clinical Microbiology and Infection* **16**(1)**:** 16-23.

[19] Lecuit M, Ohayon H, Braun L, Mengaud J, Cossart P (1997). Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infection and immunity* **65**(12)**:** 5309-5319.

[20] Marchand JB, Moreau P, Paoletti A, Cossart P, Carlier MF, Pantaloni D (1995). Actin-based movement of *Listeria monocytogenes*: actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. *The Journal of cell biology* **130**(2)**:** 331-343.

[21] Cossart P, Sansonetti PJ (2004). Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens. *Science* **304**(5668)**:** 242-248.

[22] Jensen ER, Glass AA, Clark WR, Wing EJ, Miller JF, Gregory SH (1998). Fas (CD95)-dependent cell-mediated immunity to *Listeria monocytogenes*. *Infection and immunity* **66**(9)**:** 4143-4150.

[23] Drevets DA (1999). Dissemination of *Listeria monocytogenes* by infected phagocytes. *Infection and immunity* **67**(7)**:** 3512-3517.

[24] Weinberg ED (1984). Pregnancy-associated depression of cell-mediated immunity. *Reviews of infectious diseases* **6**(6)**:** 814-831.

[25] AOAC Official Method 992.18. 2000. MICRO-ID *Listeria*. Chapter 17.10.02, pp. 141-144 In: Official Methods of Analysis of AOAC INTERNATIONAL. 17th Edition. W. Horwitz (ed.). Volume 1. Agricultural Chemicals, Contaminants and Drugs. AOAC INTERNATIONAL, Gaithersburg, MD.

[26] Zhang W, Hughes A, Wilt G, Knabel SJ (2004). The BAX PCR assay for screening *Listeria monocytogenes* targets a partial putative gene lmo2234. *Journal of Food Protection* **67**(7)**:** 1507-1511.

[27] Gouin E, Mengaud J, Cossart P (1994). The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species. *Infection and immunity* **62**(8)**:** 3550-3553.

[28] Amagliani G, Giammarini C, Omiccioli E, Brandi G, Magnani M (2007). Detection of *Listeria monocytogenes* using a commercial PCR kit and different DNA extraction methods. *Food Control* **18**(9)**:** 1137-1142.

[29] Rossen L, Norskov P, Holmstrom K, Rasmussen OF (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International journal of food microbiology* **17**(1)**:** 37-45.

[30] Wagner T, Abraham G, Baum J (1974). The roles of IgG, IgM rheumatoid factor, and their complexes in the induction of polymorphonuclear leukocyte chemotactic factor from complement. *The Journal of clinical investigation* **53**(6)**:** 1503-1511.

[31] Bille, J., Rocourt J. 2003. *Listeria* and *Erysipelothrix*, p. 461-471. In P.R. Murray, E.J. Baron, J.H. Jorgensen, M.A. Pfaller and R.H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.

[32] Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2003). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *Journal of medical microbiology* **52**(Pt 12)**:** 1065-1070.

[33] Leimeister-Wachter M, Domann E, Chakraborty T (1992). The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *Journal of bacteriology* **174**(3)**:** 947-952.

[34] Peel M, Donachie W, Shaw A (1988). Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. *Journal of general microbiology* **134**(8)**:** 2171-2178.

[35] Kohler G, Milstein C (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**(5517)**:** 495-497.

[36] Johnstone A, Thorpe R. Immunochemistry in practice. 2nd ed. Oxford: Blackwell Scientific Publications; 1987.

[37] Hassett DJ, Charniga L, Bean K, Ohman DE, Cohen MS (1992). Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. *Infection and immunity* **60**(2)**:** 328-336.

[38] Palombo G, Verdoliva A, Fassina G (1998). Affinity purification of immunoglobulin M using a novel synthetic ligand. *Journal of Chromatography B: Biomedical Sciences and Applications* **715**(1)**:** 137-145.

[39] Hutchens TW (1992). Thiophilic adsorption chromatography. *Methods in molecular biology (Clifton, N.J.)* **11:** 1-15.

[40] Webster RG (1968). The immune response to influenza virus. 3. Changes in the avidity and specificity of early IgM and IgG antibodies. *Immunology* **14**(1)**:** 39-52.

[41] Wong AH, Gottesman, II, Petronis A (2005). Phenotypic differences in genetically identical organisms: the epigenetic perspective. *Human molecular genetics* **14 Spec No 1:** R11-18.

[42] Varki A, Cummings R, Esko J, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1999. Chapter 21, Bacterial Polysaccharides.

[43] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, *et al.* (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**(6832)**:** 1099-1103.

[44] Orcutt ML (1924). THE EFFECT OF HEAT ON FLAGELLAR AND SOMATIC AGGLUTINATION. *The Journal of experimental medicine* **40**(5)**:** 627-632.

[45] Edman PH, Erik; Sillén, Lars Gunnar; Kinell, Per-Olof (1950). *Acta Chemica Scandinavica* **4:** 283–293.

[46] Kohn J, Wilchek M (1984). The use of cyanogen bromide and other novel cyanylating agents for the activation of polysaccharide resins. *Applied Biochemistry and Biotechnology* **9**(3)**:** 285-305.

[47] Madigan M; Martinko J (editors). (2005). Brock Biology of Microorganisms (11th ed.). Prentice Hall.

[48] Hay FC (1988). Rheumatoid factor: structural and genetic studies indicating novel binding sites may confer the specificity for IgG Fc. *British journal of rheumatology* **27 Suppl 2:** 157-161.

[49] Wagner T, Abraham G, Baum J (1974). The roles of IgG, IgM rheumatoid factor, and their complexes in the induction of polymorphonuclear leukocyte chemotactic factor from complement. *The Journal of clinical investigation* **53**(6)**:** 1503-1511.

[50] Waller M, Duma RJ (1972). Increased antibodies to IgG fragments: correlation with infection due to gram-positive bacteria. *The Journal of infectious diseases* **125**(1)**:** 45-53.

[51] Surolia, A., Pain, D. and Khan, M. I. (1982) Protein A: nature's universal anti-antibody. *Trends in Biochemical Science.* **7**, 74-76.

[52] Shen LL, Pernet AG (1985). Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proceedings of the National Academy of Sciences of the United States of America* **82**(2)**:** 307-311.

[53] Lampidis R, Kostrewa D, Hof H (2002). Molecular characterization of the genes encoding DNA gyrase and topoisomerase IV of *Listeria monocytogenes*. *The Journal of antimicrobial chemotherapy* **49**(6)**:** 917-924.

[54] Troxler R, von Graevenitz A, Funke G, Wiedemann B, Stock I (2000). Natural antibiotic susceptibility of *Listeria* species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri* strains. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **6**(10)**:** 525-535.

[55] DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. By C Knox, V Law, T Jewison, P Liu, S Ly, A Frolkis, A Pon, K Banco, C Mak, V Neveu, Y Djoumbou, R Eisner, A C Guo, D S Wishart Nucleic Acids Research 2011-01-01.

[56] Martindale: The Extra Pharmacopoeia, 31st ed., Reynolds, J. E. F. The Pharmaceutical Press (London, England: 1996), pp. 1529-1531.

[57] Courtieu AL, Drugeon HB, Bryskier A, Chung SS, Reynaud A (1982). [Comparative activity of cefsulodin on *Pseudomonas aeruginosa*, *Acinetobacter* and Enterobacteriaceae (author's transl)]. *Pathologie-biologie* **30**(6)**:** 432-439.

[58] Abriouel H, Franz CM, Ben Omar N, Galvez A (2011). Diversity and applications of *Bacillus* bacteriocins. *FEMS microbiology reviews* **35**(1)**:** 201-232.

[59] Warth AD (1978). Relationship between the heat resistance of spores and the optimum and maximum growth temperatures of *Bacillus* species. *Journal of bacteriology* **134**(3)**:** 699-705.