Phenotypic Characterisation of Wild Type and Mutant Strains of Adherent Invasive *Escherichia coli* and *Shigella: in vitro*, *ex vivo* and *in vivo* Models

by

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Author's Declaration

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

Signed:

Date:

Dedication

I dedicate this project to God Almighty my creator, my strong pillar, my source of inspiration, wisdom, knowledge, and understanding. He has been the source of my strength throughout this program and on His wings only have I soared. I also dedicate this work to the family of the Prophet Muhammad (on them have the best prayer and peace).

This thesis is dedicated to the spirit of my mother and my brothers (Alaa and Thaer).

This work is dedicated as well as to my kids Ahmed and the sweetheart Mohammad. I feel very grateful and humbled to have such a nice family who supported and joined me throughout my journey.

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Abstract

Crohn's disease (CD) is an inflammatory bowel disease (IBD) that is largely the result of aberrant immune responses, such as the increased production of proinflammatory cytokines including IL-1, TNF-α, IFN-γ, to microbial infections in genetically susceptible individuals. Adherent-invasive Escherichia coli (AIEC) has been implicated in CD, as has *Shigella sonnei* due to the shared inflammatory characteristics and susceptible genetic background of Shigellosis and Crohn's disease patients, with both microorganisms showing resistance to antibiotics. Resistance to antibiotics appears to be a factor in gastrointestinal diseases. Therefore, the present study sought to evaluate the key AIEC and *Shigella sonnei* virulence genes for their pathogenic roles and to address the problem of antibiotic resistance and also prevent the destruction of beneficial gut bacteria by targeting the *dsbA* gene (as one of the cardinal genes) using simple compounds such as the monoterpene, geraniol, which can inhibit the activity of bacteria rather than eradicate them.

This study employed a red lambda system to construct mutants of key virulence genes, such as *dsbA, yadA, proQ, icsA* and *mxiD* genes, in AIEC and Shigella. Murine cell line cultures *in vitro* (the RAW 264.7 macrophage cell line) and *ex vivo* (intestinal organoids derived from), were used together with *in vivo* (*Galleria* moth larvae) models to evaluate and ascertain the role of these genes in the pathogenesis of AIEC and *Shigell*a. The antimicrobial activities of geraniol were also evaluated by using the above models. Nitric oxide (NO) was estimated by measuring the production of nitrite using Griess reagent. Cell viability was assessed using MTT dye. The formation/ growth rate of biofilms was also measured via a spectrophotometer to measure the optical density. Gentamicin protection assays were also used to determine the differences between wild type and mutated bacterial strains whereby RAW 264.7 cells were stimulated with cytokines in the presence of either geraniol or L-NAME.

The *dsbA* gene, shared by AIEC and *Shigella sonnei,* was one of the key genes found in both pathogens, so the deletion of this gene in the current study was found to have

a major impact in terms of reducing bacterial virulence. However, although mutation of the *yadA* gene was not achieved in AIEC, the competitive assay found that yadA significantly reduced the death rate in moth larvae. Moreover, *Shigella sonnei* genes for the Type III secretion system (TTSS), adherence, and RNA regulation also significantly reduced virulence. Geraniol was demonstrated to be effective in inhibiting bacterial infection and biofilm formation by AIEC and *S. sonnei.* Furthermore, it was found to reduce NO production caused by AIEC infection in RAW 264.7 cells, indicating control of inflammation characteristic of CD and shigellosis. Differences in the reduction of adhesion were found between the dsbA mutant and wild-type strains for AIEC and *Shigella* in experiments using intestinal organoids directly infected. The co-culture of intestinal organoids with RAW 264.7 cells resulted in severe damage to the organoid cell structure and significant damage was also observed in response to LPS and cytokines such as TNF-α and IFN-γ. This could have led to either the death of the organoids or protection of the organoid from bacterial-induced damage when using geraniol treatment compared to L-NAME.

The data generated from these models and other experiments were consistent in that deletion of all the target genes were found to be important for virulence, with significant differences emerging between wild-type and mutated strains. This implies that these genes are important for bacterial virulence and pathogenesis in that the bacteria become harmless if they lose the genes by either mutation or inactivation. Hence, targeting these genes can lead to the inactivation of the bacteria, which means that targeting the *dsbA* gene using geraniol, for example, in competitive inhibition with glutathione can lead to the inhibition of bacterial growth. This suggests that geraniol, which was found to have antimicrobial properties against the strains used in this study, may have a role to play in modulating the hyper-inflammation feature of Crohn's disease and shigellosis.

Publication

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Abbreviations

AIEC	Adherent-invasive *E. coli*
Atg	autophagy-related protein
ATG16L1	Autophagy-related 16-like 1 gene
CARD	caspase recruitment domain
CARD15/NOD2	Caspase-recruitment domain 15/nucleotide-binding oligomerization domain-containing-2 receptor.
CD4	cluster of differentiation protein 4
CEACAM6 or CD66c	Carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross-reacting antigen) also known as (Cluster of Differentiation 66c).
CFs	Colonization factors
CpxRA	Histidine protein kinase, periplasmic stress sensor; inner membrane protein; CpxR.
DAEC	Diffusely Adherent *E. coli*
DAMP	Damage-associated molecular pattern
DsbA	Bacterial disulfide bond formation
ER	Endoplasmic reticulum
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
E-selectin	also is known as CD62 antigen-like family member

	E (CD62E), endothelial-leukocyte adhesion molecule 1 (ELAM-1).
EIEC	Enteroinvasive *E. coli*
ER-Sress/UPR	Endoplasmic reticulum-stress/ Unfolded protein respons
FAE	follicle-associated epithelium
FimH	mannose-specific adhesion located on the tip of type 1 fimbriae
FOXP3	Forkhead box P3
FPP	Farnesyl pyrophosphate
GALT	Gastrointestinal Associated Lymphoid Tissue
GGPP	Geranyl-geranyl pyrophosphate
GPP	Geranyl pyrophosphate
HIF-1a	Hypoxia-inducible factor 1-alpha
IBD	Inflammatory bowel diseases
ICAM-1	Intercellular Adhesion Molecule 1, also known as CD54 (Cluster of Differentiation 54) protein.
IECs	Intestinal epithelial cells
IFN	Interferon
IFNγ	Interferon gamma
IGF-II receptor	Insulin-like growth factor II receptor
IRGM	Immunity-related GTPase family M protein also known as Interferon-inducible protein 1 (IFI1).
LEE	locus of enterocyte effacement

Lgr5	mouse leucine-rich repeat-containing G protein-coupled receptor 5
LPF	Long polar fimbriae.
LPS	Lipopolysaccharide.
MΦ	Tissue macrophages.
M1 macrophages	Macrophages that encourage inflammation
M2 macrophages	That decrease inflammation and encourage tissue repair
MHC	Major histocompatibility complex
NCF4	Neutrophil cytosolic factor-4 gene.
NCF4	Neutrophil cyctolic factors -4 gene
NK- cells	Natural killer.
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	Nucleotide-binding oligomerization domain-containing protein
NSAIDs	Nonsteroidal Anti Inflammatory Drugs
PAMP	pathogen-associated molecular patterns
pDC	plasmacytoid DC
PMN	Polymorphonuclear neutrophil leukocytes
PRRs	polymorphisms in pattern recognition receptor
PRR	pathogen recognition receptor
PPs	Peyer's patches.
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptor

ROS	reactive oxygen species
ShET1 and ShET2	*Shigella* enterotoxin 1, 2.
siRNA	Small interfering RNA.
SRL	*Shigella* resistance locus.
ssRNA	Single-stranded RNA
STEC	LEE-positive Shiga toxin-producing *E. coli*
STING	Stimulator of IFN genes
TCR	T-cell receptor
TLRs	Toll-like receptors
TGF-beta	Transforming growth factor-beta receptor
UPEC	Uropathogenic *Escherichia coli*
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule 1
VSV	Vesicular stomatitis virus
hiPSC	Human-induced pluripotent stem cell
siRNA	Small or short interfering RNA

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1. Chapter One: The Connection between Pathogenic Bacteria and Crohn's Disease

1.1. Overview of CD

Crohn's disease (CD) is a form of inflammatory bowel disease (IBD), a chronic intestinal inflammation that can affect the entire gastrointestinal tract, from mouth to anus. It arises due to an inappropriate immune response to host-microbial interactions in a genetically susceptible individual (Abraham & Cho, 2009; Ng *et al.*, 2018; The *et al.*, 2016) (Figure 1.1). CD was initially discovered in 1913 (Mulder *et al.*, 2014), but it took another 19 years to receive the name under which it is known today. This occurred in 1932, when Crohn and his collaborators identified chronic granulomatous inflammation in 14 patients hospitalised at Mount Sinai Hospital, in New York, United States (US). In this place today, CD prevalence continues to rank the highest in the US (Aniwan *et al.*, 2017; Crohn *et al.*, 2000).

CD develops in the gastrointestinal (GI) tract. In particular, it affects the end portions of both the small and large intestines, triggering chronic inflammation (Diseases, 2014). Pain in the abdominal region, diarrhoea, fever, weight loss, anaemia, rash, arthritis, eye inflammation, and fatigue are all clinical symptoms of CD. Inflammation associated with CD is not restricted to the mucosa, but spreads throughout the thickness of the intestinal wall, involving cellular as well as non-cellular factors. As a result, granulomas and vasculitis start to develop. The thickness of the intestinal wall increases and undergoes fibrotic change due to chronic inflammation, along with the "creeping fat" arising from the mesentery. The disease can also lead to obstructions in the gut, and it increases the risk of bowel cancer (Baumgart *et al.*, 2007).

A major feature of CD is the overproduction of TH1 cytokines, including TNF-α and IFN-γ. Marks and Segal (2008) proposed that CD is caused by a malfunction in the innate immune system due to macrophages failing to secrete cytokines as they should such as IL-8 and IL-6. This leads to the development of inflammation in the colon due to the extensive microbial population (Dessein *et al.*, 2008; Marks *et al.*, 2006). It is noteworthy that autophagy and immunity display a complementary

relationship, with adaptive immune responses boosted by autophagy. At the same time, autophagy is upregulated by cytokines and receptors associated with the innate and adaptive immune system. Dessein *et al.* (2008) suggested that the presence of microbial antigens may cause the immune system to attack the GI tract, but the researchers stressed that the participation of the immune system did not warrant the classification of CD as an autoimmune disease. Despite this, Marks *et al.* (2010) and Yamamoto-Furusho and Korzenik (2006) suggested that immunodeficiency might account for CD development.

Nevertheless, the identification of more than 70 susceptible variants of Crohn's disease in the gene regions NOD2, IL23R, and ATG16L1 suggest that genetic factors are most strongly involved in the disorder (Barrett *et al.*, 2008). The most important of these participate in the processes of bacterial identification, response, and elimination in a process called autophagy. Gevers *et al.* (2014) and Zhou *et al.* (2016) reported that compared to individuals without CD, those with CD presented with changes in the gut microbiome. Additionally, studies have revealed an increase in the number of *Escherichia coli* (*E. coli*) associated with the mucosa in the ileum and the colon (Gevers *et al.*, 2014; Kotlowski *et al.*, 2007; Nishino *et al.*, 2018). These findings involved a sample group of patients who were genetically susceptible to developing CD commensal bacteria, which in other circumstances are beneficial (i.e., may serve as disease mediators instead of pathogenic agents). For example, most *E. coli* strains in the GI tract do not cause harm, but inflammation fosters the growth of pathogenic strains. These strains attach to and insert themselves into the cells lining the gut, where they undergo multiplication. Therefore, conditions such as IBD and bowel cancer are associated with an abundance of these pathogenic strains.

Since CD develops due to an aberrant response to gut bacteria, many studies have sought to identify the pathogenic agents responsible for this response (Packey & Sartor, 2009). However, bacterial species triggering inflammation, and those merely exploiting the conditions associated with inflammation have yet to be differentiated with certainty (Pineton de Chambrun *et al.*, 2008). Meanwhile, based on the findings

of (Qin *et al.*, 2010), who reported that IBD patients presented with an unusual bacterial composition in the gut, it seems reasonable to suggest that the rapid increase in harmful bacteria in the GI tract has a genetic basis.

In mammals, the gut is host to microorganisms numbering in the trillions. Most microorganisms live in symbiosis with the host; collectively they form microbiota. The human gut microbiota plays an important role in shaping the intestinal immune system in healthy people. During the first two weeks of life, a healthy microbiome is formed, which typically remains stable from that point onwards (Eckburg *et al.*, 2005; Guinane & Cotter, 2013). For example, *E. coli* has been identified as the numerically dominant inhabitants of the healthy human gut microbiota. *E. coli* plays an essential role in maintaining normal intestinal homeostasis and luminal microbiota stability through several mechanisms, including the synthesis of vitamin B and K and the metabolization of bile acids (Neut *et al.*, 2002).

An imbalance in commensal microbiota, known as dysbiosis, occurs when the number of beneficial species of bacteria decreases, while the number of harmful species increases. The dysfunctional eradication of intracellular bacteria and dysfunctional intestinal barrier make the host genetically incapable of containing commensal microbiota, and also ineffective in regulating host immunity (Agus *et al.*, 2014). When the microbial community is disrupted, opportunistic pathogenic agents, including adherent-invasive *E. coli* (AIEC), begin to proliferate. This makes intestinal inflammation more prevalent and severe in the case of CD (Agus *et al.*, 2014; Hornung *et al.*, 2018). Correlations between CD and dysbiosis are more clearly marked in mucosal biopsies (i.e. mucosa-associated bacteria populations) relative to bacterial species in the intestinal lumen (i.e. faecal samples) (Strauss *et al.*, 2011; Willing *et al.*, 2009). In fact, the significant shift in the regular gut microbial population in both experimental colitis and human IBD is associated with intestinal inflammation (Mahid *et al.*, 2007).

Classified as a pathobiont (Mazmanian *et al.*, 2008; Oberc & Coombes, 2018), AIEC has been detected in the human intestinal mucosa. It has also been identified as more

prevalent in CD patients compared to individuals without CD (Darfeuille-Michaud *et al.*, 2004; Glasser *et al.*, 2001; Rahmouni *et al.*, 2018). Owing to its particular phenotype, AIEC adheres to and invades intestinal epithelial cells, and survives and replicates in macrophages (Darfeuille-Michaud, 2002; Migliore *et al.*, 2018).

CD prevalence is highest in the Scandinavian (Bjornsson & Johannsson, 2000), where 8-10 in every 100,000 individuals receive a diagnosis each year (Lapidus, 2006). In the UK and North America, Caucasians are more likely to develop CD compared to people of black African origin (Gunesh *et al.*, 2008). Among Caucasians, Jewish people are particularly susceptible to CD. Nevertheless, such statistics should be approached with care, because there is a possibility that they may not be indicative of the actual prevalence of CD; instead, they may only show prevalence measured according to the number of people who were diagnosed and hospitalised with IBD. Additionally, changing rates of CD prevalence, along with regional trends, are worth considering. For example, from the 1990s onwards, CD prevalence began to increase in Asia, an area with historically low CD prevalence (Yang *et al.*, 2001). Amre *et al.* (2007) suggested that a diet rich in high-fat or refined foods may contribute to the development of CD.

Although CD can develop at any age, the highest prevalence is associated with individuals between the ages of 15 and 30. Susceptibility appears to be somewhat higher in females with a positive family history of CD, which suggests that there may be a genetic difference in CD when diagnosed at a more advanced age (Bayless *et al.*, 1996; Polito *et al.*, 1996). The main lifestyle factors that could lead to the development of CD include cigarette smoking and the use of non-steroidal anti-inflammatory drugs (NSAIDs). Cosnes (2004) drew attention to the importance of smoking cessation as a strategy for reducing CD risk, as well as the risk of other serious diseases. The researcher noted that this would also reduce the need for surgical intervention. Meanwhile, NSAIDs, including ibuprofen, Motrin IB, Aleve (naproxen), Anaprox, Voltaren, and Solaraze, may aggravate CD by making it easier for harmful substances and organisms to penetrate the bowel mucosa. This is

possible due to their influence on the aggregation of neutrophils and smooth muscle contraction (Orchard & Jewell, 1997). Nevertheless, it is not certain whether later manifestations of CD are a result of the use of NSAIDs to treat initial CD symptoms occurring outside the gut (Wolfe *et al.*, 1999).

Caecum and colon are the primary targets for ulcerative colitis while Crohn's disease affects all parts of GIT. Increase CRP levels as well, less useful as a marker for ulcerative colitis than the disease of Crohn's. The combination of bloody diarrhoea in a young adult with poor response to antibiotic treatment is an important indication of the potential for undiagnosed ulcerative colitis that differentiated from the treatment-responding infectious colitis and Crohn's disease where the diarrhoea is less common. Crohn's disease also fails to respond to surgical therapy while ulcerative colitis reacts. Studies of chronicity characteristics such as distorted crypt architecture, Paneth cell metaplasia and increased plasma cell/lymphocyte infiltration may help diagnose ulcerative colitis, whereas submucosal or transmural inflammation common; deep fissuring ulcers, fistulas; patchy changes; granulomas in Crohn's disease compared to infectious colitis (Tanaka *et al.*, 1999; Waugh *et al.*, 2013). Infectious colitis mainly resulted from *Shigella* species, *Campylobacter*, *Salmonella* spp, and *Aeromonas* species (Lin *et al.*, 2017; Talan *et al.*, 2001).

It is only in recent years that the high mortality rate associated with CD has been successfully reduced, and this can be attributed to medical developments and transformations in clinical practice. Many of these advancements have improved CD management (Card *et al.*, 2003). Nevertheless, although fewer patients die from CD, it continues to be a difficult condition that must be treated and managed from the moment of diagnosis (usually early adulthood), and which may require prolonged stays in hospital with potential surgical intervention.

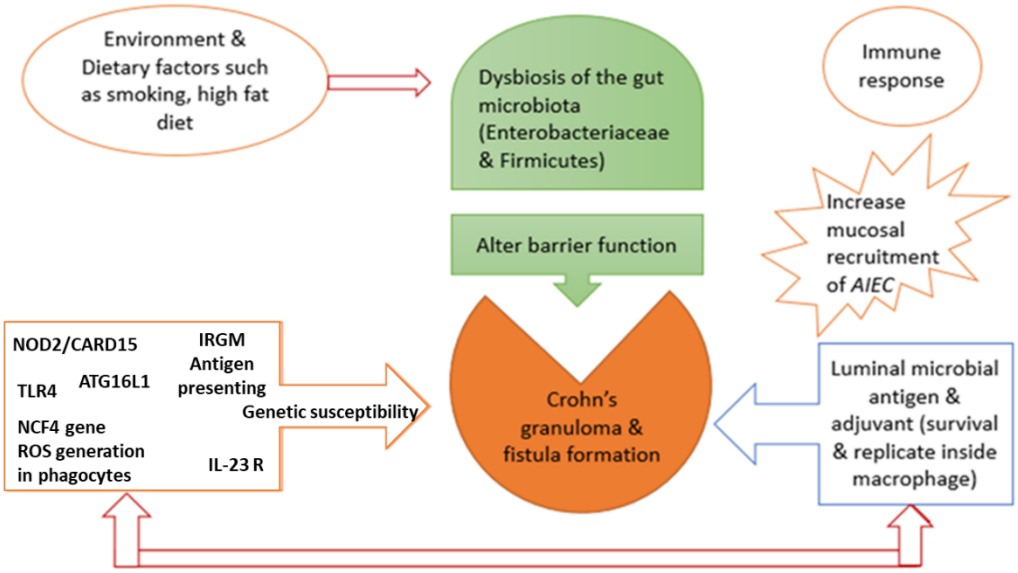


Figure 1. 1. Schematic representation of CD development. AIEC: adherent invasive *Escherichia coli*; ATG16L1: autophagy-related 16-like 1; $CARD_{15}$/NOD_2: caspase-recruitment domain 15/nucleotide-binding oligomerization domain-containing-2 receptor; IL-23R: interleukin-23 receptor; IRGM: immunity-related GTPase M; LPS: lipopolysaccharide; NCF4: neutrophil cytosolic factor-4 gene; PRR: pathogen recognition receptor; ROS: reactive oxygen species; TLR4: toll-like receptor 4.

Less-than-ideal lifestyle involving the consumption of a diet rich in fat, smoking, and stress as well as genetically susceptible individuals that promote the growth of harmful bacteria to the detriment of beneficial bacteria, resulting in chronic inflammation, pathogenic bacteria proliferation, and aggravation of infection adopted from (Tawfik *et al.*, 2014).

1. 2. Genes Associated with Bacterial Autophagy in Humans:

Although CD is not transmitted from parents to children, it is linked to over 30 distinct genetically susceptible loci (Barrett *et al.*, 2008). The bacterial invasion may activate the innate immunity, keeping the host alive until the adaptive immunity is activated. Research indicates that, in the context of innate immunity, autophagy (xenophagy)

plays a vital function, instantly detecting, capturing, and eradicating pathogenic agents (Gutierrez *et al.*, 2004; Nakagawa *et al.*, 2004).

Initially, doubts existed about whether the autophagy genes, ATG16L1, and immunity-related GTPase M (IRGM) encoded proteins that can contribute to the innate immune response by regulating the formation of autophagy in response to intracellular pathogens, which were proposed as novel loci of CD vulnerability. Nevertheless, as it became clear that CD was related to these genes, as well as to NOD2 (CARD15) polymorphisms where NOD2 protein plays an important role in activating the nuclear factor-kappa-B, which in turn regulates multiple gene activity, including genes that control immune responses and inflammatory reactions. NOD2 protein also plays a role in a process called anti-invader autophagy and in recycling worn-out cell parts, breaking down certain proteins, and cell self-destruction when they are no longer needed in the process called apoptosis (Park & Jeen, 2019). It was concluded that, in CD immune-pathogenesis, intracellular bacteria were erroneously detected and managed. Autophagy also protects cells by hindering the proliferation of intracellular bacteria (Birmingham *et al.*, 2006). This is why dysfunctional autophagy leads to persistent infections, including those caused by *Salmonella typhimurium, Streptococcus pyogenes,* and *Mycobacterium tuberculosi*s (Kuballa *et al.*, 2008). The autophagy genes ATG16L1 and IRGM, which are variants in the autophagy genes ATG16L1 and IRGM, are present in CD, with the Thr300Ala substitution in ATG16L1 being associated especially with ileal CD (Hampe *et al.*, 2007).

A genome-wide study conducted in (Consortium, 2007) was the first to establish a correlation between IRGM and CD. As a member of the GTPase family (p47 GTPases) associated with antimicrobial immunity, IRGM can bolster autophagy by activating mitochondrial depolarisation, as well as by increasing the production and death of ROS (Figure 1.2) (Singh *et al.*, 2010). In addition to the elimination of pathogens, autophagy also stimulates adaptive immunity by transporting cytosolic antigens to autolysosomes, where they are broken down and integrated into major

histocompatibility complex class II (MHC II) molecules (Crotzer & Blum, 2005). Subsequently, peptide-MHC II complexes are presented on the surface of the cells, where they are detected by T-cells through their T-cell receptor (TCR) (Schmid *et al.*, 2007). Thus, it can be surmised that a valuable source of bacterial antigens for MHC II presentation is supplied through the autophagy-based breakdown of intracellular pathogens. Specialised cells with antigens, including dendritic cells, macrophages, and especially epithelial cells that are not wholly capable of phagocytosis, may benefit from the provision of antigens through autophagy.

In comparison to the Toll-like receptor (TLR), which plays a role in the surface detection of pathogenic agents, the receptors of the innate immunity are present in the cell cytosol. The family of NOD proteins is among these receptors. CARD15 encodes the NOD2 receptor (Hugot *et al.*, 2001). It has been established that three single nucleotide polymorphisms (SNPs) of the gene NOD2/CARD15 are independently associated with the development of CD in Caucasians (Leong *et al.*, 2003). The gene NOD2 was later renamed CARD15, reflecting the two domains of caspase recruitment observed at the gene's N-terminus. NOD2 shows sero-reactivity towards the microbes involved in CD, and therefore is of central importance in the inflammatory response targeting the peptidoglycan constituents of the bacteria (Hayashi *et al.*, 2001). The bacterial antigen that binds to the TLR on the surface of monocytes and macrophages, as well as to the cytoplasmic NOD2, is responsible for activating pro- and anti-inflammatory cytokines via the nuclear factor kappa-light-chain-enhancer of the activated B-cell (NF-κB) pathway (Abreu *et al.*, 2002).

CD is often accompanied by NOD2 mutations in the leucine-rich part of the NOD2 molecule, which plays a role in the isolation of a muramyl-dipeptide. Contrastingly, the detection of bacteria depends greatly on NOD1, which is geared towards the peptidoglycan-muramyl tripeptide of Gram-negative bacteria (Girardin *et al.*, 2003). In addition to immune cells, Paneth cells also express NOD2, and this protein should therefore not be ignored (Lala *et al.*, 2003). Studies have shown that NOD1 senses a large number of Gram-negative bacteria while NOD2 senses Gram-positive and

Gram-negative bacteria where it has been found that both NOD1 and NOD2 function synergistically to change the responses to certain pathogens (Moreira & Zamboni, 2012). For instance, double-deficient mice NOD1 and NOD2 showed a substantial reduction in inflammatory cytokine development and an increase in mucosal tissue bacterial colonisation in a *Salmonella* model of colitis (Moreira & Zamboni, 2012).

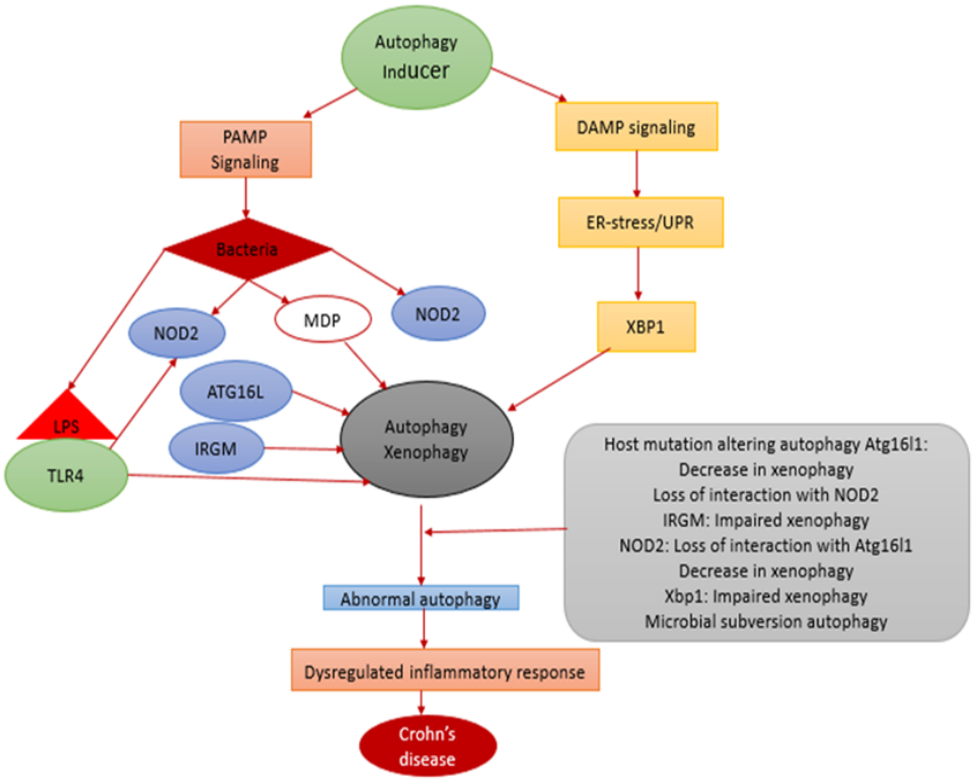


Figure 1. 2. Crosstalk between autophagy and innate immune system in CD. Toll-like receptors (TLRs) are the recognition receptors found on the surface of macrophage and dendritic cells (sentinel cells). The TLRs react to the microbes that have passed through barriers, including to the intestinal mucosa, facilitating an immune cell response adopted from (Muzes *et al.*, 2013). Among the immune and inflammatory signals that ensure positive regulation of autophagy, where autophagy has been triggered in infected epithelial cells as a result of upregulation of murine Irgm1 by IFN-γ and LPS, are bacterial LPS, TH1 cytokine IFN-γ and TNF family members (Gutierrez *et al.*, 2004; Xu *et al.*, 2007). Therefore, this amplification process, which creates a link between autophagy and immunity, may

potentially eliminate eradicate the infection, but the disturbance leads to Crohn's disease (Schmid *et al.*, 2007).

1. 3. Microbiota and CD

The microbiota refers to an "ecological species of commensal, symbiotic, and pathogenic micro-organisms found in and on all multicellular organisms studied so far, from plants to animals" (Group *et al.*, 2009). The microbiota has been identified as playing an essential role in the host's immunological, hormonal, and metabolic homeostasis (Salvucci, 2016). Furthermore, it can be described as the ensemble of microorganisms involved in immunity support (Round & Mazmanian, 2009), production of substrate-digesting enzymes (El Kaoutari *et al.*, 2013), inhibition of harmful microorganisms (O'Hara & Shanahan, 2006) and other key functions. The gut microbiota consists of several species of microorganisms, including bacteria, yeast, and viruses.

A normal gut microbiota primarily comprises bacteria of the phyla *Firmicutes, Actinobacteria*, and *Verrucomicrobia* (Jandhyala *et al.*, 2015). Microbiota diversity increases in complexity from birth to around one year of age (Backhed *et al.*, 2015; Koenig *et al.*, 2011). This initially occurs as a function of diet, and it is afterward unaffected by external disruption (Dethlefsen & Relman, 2011; Wu *et al.*, 2011). However, microbiota composition differs from person to person (Backhed *et al.*, 2012; Qin *et al.*, 2010; Turnbaugh *et al.*, 2009). Age, genetics, diet, and drugs are important influencers of the microbiota (Maier *et al.*, 2018; Yatsunenko *et al.*, 2012; Zuo *et al.*, 2018). Microbiota disruption diminishes biodiversity in terms of α diversity and species richness, promoting *Gamma-proteobacteria* and reducing *Firmicutes* and *Bacteroides* abundance (The microbe diversity within a given body habitat can be represented by its richness and evenness, I.e. the number of species within the sample relative to the species abundance (alpha-diversity), with a high diversity

associated with a healthy state). Loss of microbial richness may be associated with amino acid synthesis, cellular junction integrity, and inflammatory response, indicating failure of the epithelial barrier may partially explain the symptoms of IBS (Frank *et al.*, 2011; Lozupone *et al.*, 2012; Morgan *et al.*, 2012; Sartor, 2008; Tamboli *et al.*, 2004).

The reduced diversity reported in CD studies, including Manichanh *et al.* (2006) and Dicksved *et al.* (2008), was correlated with prevalent temporal taxa instability in IBD (Martinez *et al.*, 2008), as well as with inflamed tissue in the same patient with lower diversity compared to non-inflamed tissue (Sepehri *et al.*, 2007).

In Qin *et al.* (2010), the researchers reported that IBD patients presented with an unusual bacterial composition in the gut, and so it could be argued that the rapid increase in harmful bacteria in the GI tract had a genetic basis in these patients. Contrastingly, in patients with genetic susceptibility to CD development (Figure 1.1), commensal bacteria in other circumstances are beneficial, and they may serve as disease mediators rather than pathogenic agents. For example, most *E. coli* strains in the GI tract do not cause harm, but inflammation fosters the growth of pathogenic strains. These strains attach to and insert themselves into the cells lining the gut, where they undergo multiplication. Therefore, conditions such as IBD and bowel cancer are associated with an abundance of these pathogenic strains.

CD is associated with dysbiosis (Oberc & Coombes, 2015; Ricanek *et al.*, 2012), as well as diminished numbers of *Firmicutes, Proteobacteria* proliferation, and fungal composition changes (Liguori *et al.*, 2016). Naftali *et al.* (2016) also reported microbiota discrepancies among CD patients with the ileal and colonic disease, which may be due to a CD-auxiliary epiphenomenon (an additional symptom or condition that appears alongside or in parallel to a primary disease). Diarrhoea (Youmans *et al.*, 2015), enteral nutrition (Quince *et al.*, 2015), antibiotics (Hashash *et al.*, 2015; Zaura *et al.*, 2015) and iron supplementation for anaemia (Lee *et al.*, 2017) may all impact the microbiota of CD patients.

Studies on both humans and mice have reported that *Enterobacteriaceae* proliferate in the inflammatory environment created by IBD (Lupp *et al.*, 2007). Contrastingly, ileal CD (Darfeuille-Michaud *et al.*, 2004) and UC (Sokol *et al.*, 2006) have been associated with *E. coli,* especially AIEC strains, with a greater presence in mucosa than faeces (Chassaing & Darfeuille-Michaud, 2011). Bacterial numbers (e.g. *Escherichia*/*Shigella*) can be controlled, and inflammation alleviated, with the anti-inflammatory drug mesalamine (Benjamin *et al.*, 2012; Morgan *et al.*, 2012).

Lactobacilli, Bifidobacteria, and *Faecalibacteria* are among the bacterial species responsible for protecting against IBD-related inflammation. This occurs through the activation of anti-inflammatory cytokines (Sokol *et al.*, 2008), as well as the restriction of inflammatory cytokine (Llopis *et al.*, 2009). IBD patients have low levels of the anti-inflammatory *Faecalibacterium prausnitzii*, which can also increase post-surgery CD relapse (Sokol *et al*., 2008, 2009). However, a return to normal levels has been reported to prevent UC (Varela *et al.*, 2013), and ileal CD is associated with *E. coli* proliferation (Willing *et al.*, 2009).

The gut microbiota is significantly affected by diet. A Western diet has been shown to increase the risk of IBD and other diseases (Agus *et al.*, 2016; Albenberg & Wu, 2014; Conlon & Bird, 2014; Ley *et al.*, 2008). This occurs through an increase in the *Bacteroides-Firmicutes* ratio, as well as an enhanced vulnerability to AIEC infection (Agus *et al.*, 2016). Shivashankar *et al.* (2017) reported that the incidence of CD and UC increased through the consumption of common Western dietary products, namely pineapples and coffee products, respectively. However, the mechanisms underpinning enhanced IBD susceptibility are unknown. Agus *et al.* (2016) observed that the mouse microbiota was permanently depleted by a low-fibre diet.

Considering the CD-related microbiota discrepancies, and since CD develops mainly in the gut areas with the great abundance of bacteria, treatments have sought to change the microbiota with the use of materials (prebiotics), thereby promoting beneficial bacteria and live microorganisms (probiotics). However, most of the proposed initiatives have been unsuccessful (Benjamin *et al.*, 2011; Bernstein, 2014).

1. 4. Diseases similar to CD

CD is a chronic IBD. Recently, it was found that CD is caused by bacterial infection with biofilm-forming AIEC pathogenic strains of *E. coli* (Martinez-Medina *et al.*, 2009a; Tawfik *et al.*, 2014). The disease shares some similarities to dysentery in terms of the association with the NOD2/CARD15 and ATG16L1 mutations that hinder autophagy (Cuthbert *et al.*, 2002; Prescott *et al.*, 2007; Travassos *et al.*, 2010). Despite lacking the *Shigella* virulence plasmid, AIEC strains can invade gut epithelial cells (Darfeuille-Michaud *et al.*, 2004; Martin *et al.*, 2004), spread via microfold cells (Chassaing & Darfeuille-Michaud, 2011; Chassaing *et al.*, 2011; Dogan *et al.*, 2014; Roberts *et al.*, 2010), survive and replicate within macrophages, and activate TNFα (Bringer *et al.*, 2007; Mpofu *et al.*, 2007; Subramanian *et al.*, 2008b)

Antibiotics do not resolve chronic immune-related conditions, and they can actually cause severe complications (Meropol *et al.*, 2008; Selby *et al.*, 2007). Antibiotics may even engender chronic inflammatory disorders such as asthma and CD in infants (Hviid *et al.*, 2011; Jedrychowski *et al.*, 2011). An immune response is altered by the antibiotic-induced changes in microbiota composition (Brandl *et al.*, 2008).

Rather than inducing uniform mucosal damage, most gastrointestinal infections engender focal ulceration sites in the ileocaecal area of the bowel (Puylaert *et al.*, 1997). Furthermore, the stochastic nature of pathogenic agent infections (Rock *et al.*, 2014) means that there is significant variation in the age of onset and outcomes. These are informed by factors such as infection seriousness, ulceration degree, amount of bowel contents with tissue accessibility, and the strength of innate immunity.

Seeking to prove that CD is caused by infection, various empirical studies have attempted to transfer a CD agent from human patients to wild type or immunodeficient mice, with inconclusive results (Segal, 2016). Researchers have especially focused on proving that human CD is caused by the same agent responsible

for Johne's disease in cattle (Hermon-Taylor *et al.*, 2000; Wu *et al.*, 2007), without success so far (Chiodini *et al.*, 2012; Gitlin *et al.*, 2012). Johne's disease, also called Paratuberculosis, is a chronic, infectious, granulomatous infection of cattle's intestinal tract and other domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Fecteau, 2018).

Gut microbiota disruption is related to many conditions, including IBD, and specific bacterial species are involved, including AIEC in CD. The *E. coli* AIEC strains have been extensively investigated regarding CD pathogenesis (Agus *et al.*, 2014; Rolhion & Darfeuille-Michaud, 2007). CD lesions are similar to those associated with ileal and colonic infections (Colgan *et al.*, 1980; Puylaert *et al.*, 1997; Segal, 2016).

Infections with different organisms are related to IBD relapses (Mylonaki *et al.*, 2004). However, the term is misleading because such infections might cause new lesions to form. Furthermore, these secondary infections might be the result of standard immunosuppressive treatments.

1. 5. Pathogenesis

CD is a highly complex disease, and its development is still not fully understood. Nevertheless, it is agreed that CD arises when the immune system responds to bacteria in the gut in individuals who are genetically prone to this kind of aberrant response. Three major perspectives have been put forth in the literature to elucidate the connection between bacteria and CD pathogenesis: firstly, dysbiosis (the balance of beneficial and harmful bacteria becomes destabilised); secondly, the intestinal barrier and immune response malfunction, thereby causing considerable bacterial translocation; and thirdly, the resistance of pathogens (Figure 1. 1).

The surface of the intestinal mucosa continuously comes into contact with gut bacteria. As a result, it is the main access point for opportunistic pathogenic bacteria. Therefore, it is vital to preserving homeostasis at the luminal surface of the internal microbial interface. The intestinal layer separates the intestinal lumen from the inner

milieu, and it is made up of columnar epithelial cells. These primarily include enterocytes, as well as entero-endocrine cells, goblet cells, and Paneth cells. The function of this layer is to allow the passage of water, ions, and nutrients, and to protect the mucosal surface from bacterial invasion (van der Flier & Clevers, 2009).

The regulation of the inflammatory response and the preservation of the integrity of the intestinal epithelium is achieved by immune-tolerance and innate immunity. These rely on the mechanisms of IgA production, activation of antimicrobial peptides to eliminate bacteria, and functional autophagy. It is believed that when these mechanisms do not function properly, they induce the aberrant immune reactions associated with CD (Figure 1. 3). Defensins and other antimicrobial molecules generated by the intestinal epithelium kill both commensal and pathogenic bacteria. They bind to and penetrate the membrane of microbial cells, making it porous, which thus leads to the loss of Ca^{++} entry essential ions and nutrients (Jarczak *et al.*, 2013; Kagan *et al.*, 1990). In humans, α-defensins are produced by Paneth cells and neutrophils, while β-defensins are produced by epithelial cells (Jarczak *et al.*, 2013).

Activation of receptors involved in the detection of extracellular and intracellular bacterial components (TLR and NOD receptors, respectively) initiates the biosynthesis of defensins. As a result, bacteria coming into contact with the intestinal epithelium are destroyed (Kaser *et al.*, 2010). Research on murine models indicates that defensins are involved in regulating the composition and density of bacterial populations (Salzman *et al.*, 2010). Moreover, Wehkamp *et al.* (2005) provided evidence to suggest that the infectious aetiology is related to genetic susceptibility. This is because Paneth cells produced lower amounts of α-defensins in the ileal CD, particularly when NOD2 mutations occurred as well. Contrastingly, β-defensins were produced in lower amounts in the case of colonic CD.

As has already been suggested, inadequate synthesis of antimicrobial proteins disrupts the normal functioning of the intestinal barrier. In turn, this may lead to chronic inflammation in CD. Chronic tissue swelling, damage, and ulceration occur

due to the inflammatory toxins produced by white blood cells when the lining of the intestine is invaded by bacteria. Whilst the empirical findings are not clear, there is a high probability that this kind of aberrant immune response is determined by a specific pathogenic agent in individuals genetically prone to CD.

Shigella and enteroinvasive *E. coli* are pathovars with similar virulence attributes (Schroeder & Hilbi, 2008). When they reach the colon, they establish an infection. *Shigella* species do not penetrate the epithelial barrier; rather, they exploit microfold (M) cells, which are specialised epithelial cells that the bacteria access through the basolateral pole (Mounier *et al.*, 1992).

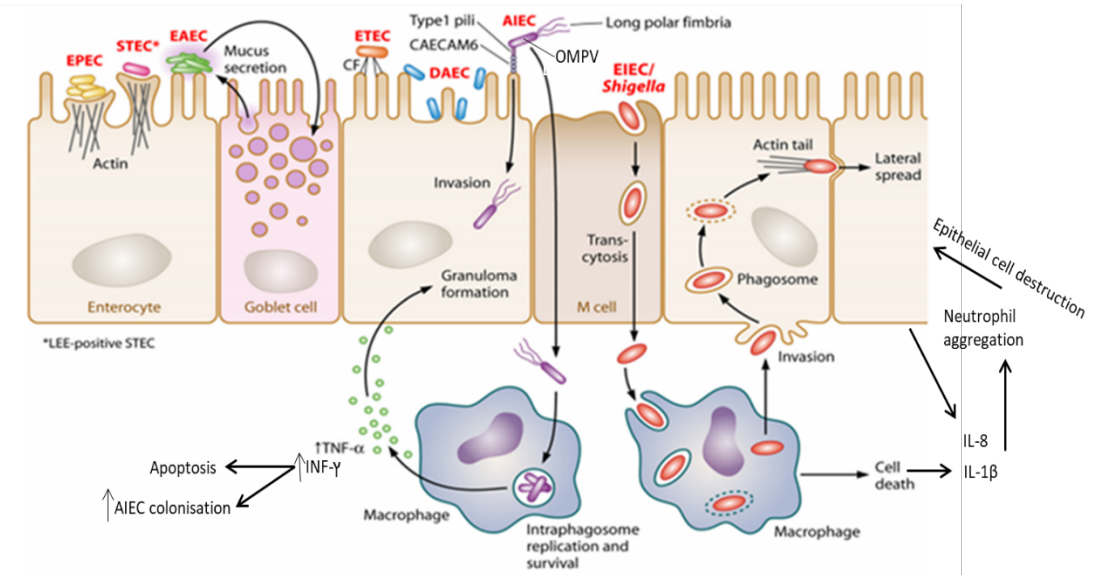


Figure 1. 3. The attachment mechanism of enteric *E. coli*. The diseases caused by *E. coli* differ according to the route of attachment to epithelial and mucosal cells in the intestine. For example, AIEC has multiple actions, including colonisation of the intestinal mucosa in CD, intrusion of epithelial cells, and proliferation in macrophages. By contrast, Enteroinvasive *E. coli* (EIEC) and *Shigella* invade the submucosa, exploiting M cells to penetrate the intestinal epithelial tissue. This leads to the destruction of macrophages, after which they undertake a basolateral invasion of colonocytes and lateral diffusion adopted from (Croxen *et al.*, 2013)

1. 5. 1. Adherent invasive E. *coli* pathogenesis

E. coli strains are genetically and phenotypically diverse (Galardini *et al.*, 2017). As a result, they are extremely significant in medicine and medical research (Bergey *et al.*, 1984). According to their serological and virulence features, there are various forms of enteric *E. coli* (EC), including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), as well as the recently isolated adherent-invasive *E. coli* (AIEC) (Nataro & Kaper, 1998).

Initially identified by Boudeau *et al.* (1999), the AIEC pathovar is now considered one of the main bacterial contenders for CD development. It includes *E. coli* strains that exhibit such properties as the *in vitro* capability of epithelial cell attachment and invasion, as well as survival and replication in macrophages (Conte *et al.*, 2014; Martinez-Medina & Garcia-Gil, 2014; Simpson *et al.*, 2006). This can occur without causing cell apoptosis or triggering the production of tumour necrosis factor-alpha (Glasser *et al.*, 2001), as well as unknown invasive determinants (Darfeuille-Michaud *et al.*, 2004). The AIEC pathovar has not been associated with any specific virulence factors to date are detectable only phenotypically, but it may be closely correlated with extra-intestinal *E. coli* (ExPEC) pathovar. This is because a large number of virulence-related genes in AIEC strains are similar to those in ExPEC strains (Baumgart *et al.*, 2007; Martinez-Medina *et al.*, 2009a).

One mechanism through which pathogens such as AIEC, EPEC, and enterohaemorrhagic *E. coli* manage to colonise the intestinal epithelium involves translocation across the M cells that line the epithelium near follicles. (Nguyen *et al.*, 2015) emphasised that genetic susceptibility factors should be taken into account in the literature, principally because they influence bacterial behaviour in the intracellular medium. Moreover, according to O'Brien *et al.* (2017), the capacity for bacterial replication intracellularly is more significant than adhesion to and invasion of macrophages. This stems from the fact that mutations in genes associated with

macrophage function may help bacteria to survive in macrophages, which implies that malfunctioning macrophages may serve as bacterial repositories.

Type I pili display mannose-related interaction with the glycoprotein CEACAM6. To some extent, they help pathogens attach to intestinal epithelial cells (Barnich *et al.*, 2007; Carvalho *et al.*, 2009). The likelihood of excessive AIEC colonisation is greater in patients with the ileal CD due to overexpression of CEACAM6. Most *E. coli*, including harmless strains, have type I pili, but FimH adhesin variants are typically exhibited by AIEC strains. This enables their affective attachment to intestinal epithelial cells (Dreux *et al.*, 2013).

Pathogens also rely on flagella to attach to and invade intestinal epithelial cells. In polarised intestinal epithelial cells, flagella trigger the production of the pro-inflammatory cytokine IL-8 and chemokine CCL20. These prompt macrophages and dendritic cells to aggregate at the infection site (Eaves-Pyles *et al.*, 2008; Subramanian *et al.*, 2008a). CEACAM6 is expressed when macrophages and lymphocytes produce IFNγ and TNFα, which boosts AIEC colonisation. HIF-1a production is stimulated and the traditional NF-κB pathway is activated when LF82 type I pili and flagella attach to CEACAM6 and TLR5, respectively, in intestinal epithelial cells (Mimouna *et al.*, 2011). Inflammation and vascularisation are promoted, as cooperation is established between these molecules for the regulation of the transcription of IL-8 and pro-angiogenic factors such as vascular endothelial growth factor.

According to Stevens *et al.* (2013), the intermediate filament vimentin, which is expressed on the mesenchymal cell surface, is an AIEC receptor. The interaction between the vimentin repeats abundant in leucine and NOD2 in the intracellular medium results in the mobilisation of these proteins at the plasma membrane. Adequate NOD2 functionality in terms of antigen detection, NF-κB activation, and autophagy depends on such mobilisation. No interaction occurs between the NOD2 variants L1007fs and R702W (two of three major variants of NOD2 risk which are R702W, G908R, and L1007fs), resulting in NOD2 feature reduction or loss. These are

present within the cytosol in individuals diagnosed with CD. Due to this lack of interaction, the inflammatory response does not manifest properly, autophagy is not activated effectively, and AIEC related to CD are not controlled adequately. Overall, identification of AIEC appears to be heavily dependent on NOD2 and vimentin, and AIEC host colonisation may be impaired if these proteins carry polymorphism.

Low *et al.* (2013) drew attention to a novel interplay between host and microbes based on the participation of a bacterial chitinase and a human chitinase that helps LF82 attach to intestinal epithelial cells. Chitinases are enzymes responsible for the hydrolysis of a long-chain N-acetylglucosamine polymer called chitin. Low and his group demonstrated that specific polymorphisms in two chitin-binding domains displayed by LF82 and additional pathogenic *E. coli* are necessary for interaction with an N-glycosylated asparagine associated with the human chitinase CHI3L1 (Low *et al.*, 2013). Tran *et al.* (2011) note that the deletion of chitin A (chiA) in the AIEC LF82 strain significantly reduces their adhesion to IECs as compared with that of wild-type AIEC LF82 bacteria. This data shows that ChiA plays a role as a virulence factor in AIEC and can facilitate adhesion of bacteria to mucosal tissues.

To invade intestinal epithelial cells, LF82 relies on outer membrane vesicles (OMVs) in which the transmembrane protein OmpA is found (Rolhion *et al.*, 2010; Rolhion *et al.*, 2005). This protein attaches to the localised stress response chaperone Gp96, which occurs in the endoplasmic reticulum, and is overexpressed on the apical surface of epithelial cells in the ileum of CD patients. Evidence indicates that the fusion of OMVs with host cells leads to the discharge of yet unknown bacterial effectors, which are implicated in invasion-related processes of actin polymerisation and microtubule mobilisation. Furthermore, as observed by (Miquel *et al.*, 2010), interaction with Gp96 is improved by point mutations in the OmpA sequence of LF82 and additional B2 strains. The excessive expression of Gp96 in the ileum enhances CD patients' risk of AIEC infection. Apart from OmpF/C and OmpA, it is not known what other components make up OMVs and possible effector proteins.

Macrophages envelop AIECs that manage to penetrate the lamina propria. Rather than moving to the cytoplasm, intramacrophage LF82 triggers the development of a large-sized vacuole, known as a phagosome, which merges with lysosomes. This implies that AIEC bacteria are capable of replicating under conditions of low pH and oxidative stress, and in the presence of active proteolytic enzymes and antimicrobial compounds. In fact, *in vitro* research conducted by (Bringer *et al.*, 2006a) revealed that AIEC LF82 bacteria could only replicate in an acidic medium. Moreover, to survive and replicate inside macrophages, the bacteria were observed as being dependent on the protease HtrA (chaperon), as well as the thiol-disulphide oxidoreductase DsbA (Bringer *et al.*, 2005; Bringer *et al.*, 2007). Given that the isogenic mutants of these proteins did not thrive in an environment with low pH and scarce nutrients and given that the proteins were overexpressed in LF82 when macrophages were infected, a connection was established between these proteins and LF82 resistance to the stress conditions in phagolysosomes.

Furthermore, TNFα production increases when LF82 replicates constantly in macrophages without triggering cell apoptosis (Glasser *et al.*, 2001). This may elucidate the development of inflammation and granuloma in the gut of CD patients, evidence for which has been provided by *in vitro* research (Carvalho *et al.*, 2008; Meconi *et al.*, 2007). Additionally, Dunne *et al.* (2013) proposed active LF82 involvement in deferring the death of infected macrophages and dendritic cells. It was observed that LF82 infection not only induced changes in the function of the protease caspase 3, which is crucially involved in apoptosis, but also accelerated the breakdown of the protease within proteasomes leading to control apoptosis by AIEC.

1. 5. 2. *Shigella* pathogenesis

Comprising several events, *Shigella* pathogenesis depends on the exploitation of the M cells of the gastrointestinal-associated lymphoid tissue (GALT), which leads to the penetration of the colonic mucosa. This is followed by the invasion of epithelial cells, which are manipulated to generate pro-inflammatory mediators (e.g. interleukin 8) that aid bacterial invasion by promoting a powerful inflammatory reaction. Most of the 27 genes required for *Shigella* invasion of epithelial cells are present in two neighbouring operons, which are orientated in opposite directions on the large virulence plasmid (Parsot & Sansonetti, 1996). The secretion or translocation of bacterial proteins in the host cell cytosol is enabled by the *Mxi-Spa* operon, which encodes a type III secretion apparatus (Figure 1. 4). This also permits the penetration of epithelial cells encoded on a 213 kb plasmid, which only virulent *Shigella* and EIEC strains possess. The survival of *Shigella* spp such as *S. flexneri* in the acidic medium of the stomach is ensured by highly efficient systems of acid resistance, which also contributes to the infectious nature of the bacteria at a low dose (Gorden & Small, 1993). Moreover, as demonstrated by Islam *et al.* (2001), *Shigella* bacteria interfere with antimicrobial peptides, which are produced continuously by intestine mucosal surfaces through reducing the expression of cathelicidin LL-37 (LL-37) and human β-defensins (HBD-1) antibacterial peptides to the escape-immune strategy. Notably, this is also used by the pathogens that cause watery diarrhoea.

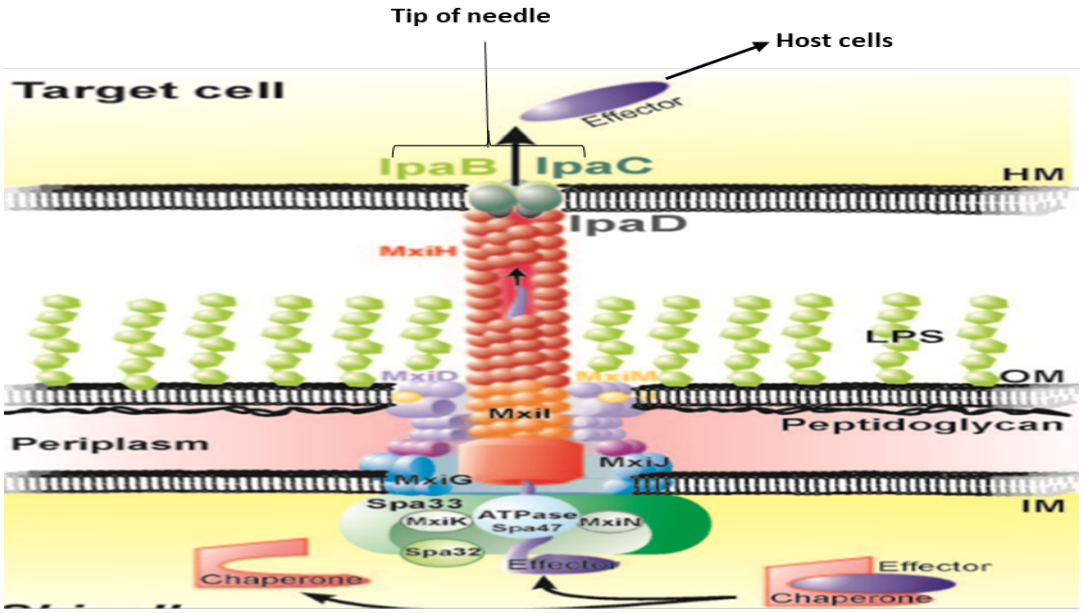


Figure 1. 4. Structure of *S. flexneri Mxi-Spa T3SS*. This operon comprises a basal body with seven rings that extend over the bacterial IM, periplasm, and OM. Affixed to a socket, the hollow needle projects from the basal body to the surface of the bacteria. The *IpaB-IpaC* translocon membrane insertion at the needle tip guided by IpaD is induced by interaction with host cell membranes (HM). The cytoplasmic C ring completes the T3SS and consists of proteins driving transport and facilitating the detection of substrates, chaperone release, and substrate unfolding adopted from (Schroeder & Hilbi, 2008).

Bacteria trigger an infection once they are in the colon. *Shigella* can manipulate tight junction proteins expressed by epithelial cells, enabling bacteria to pass into the submucosa in a paracellular manner. In response to *Shigella* invasion, PMN cells recruited by IL-8 and IL-1β establish gaps between epithelial cells, through which *Shigella* can transmigrate into the submucosa. Bacteria transcytosis of endocytic M cells occurs, releasing them into an intraepithelial pocket filled with lymphocytes and macrophages B and T, where macrophages phagocytosis of the bacteria. *Shigella*

flees from the phagosome and induces apoptosis of the macrophage, where IL-1β is released by the apoptotic macrophage.

Submucosal *Shigella* contacts the basolateral membrane of epithelial cells and stimulates protein secretion through its type-III secretion mechanism, where proteins chaperoned in the *Shigella* cytosol are secreted into the cytoplasm of the epithelial cell through a pore created by IpaB and IpaC. This induces cell surface extensions around the bacterium, which pushes the epithelial cell to take *Shigella* in a vacuole. As a result, IpaB and IpaC lyse the vacuole, releasing *Shigella* into the cytoplasm of the epithelial cell. Just one pole of the bacterium shows the *Shigella* protein, *IcsA*, forming a polymerised actin tail behind the bacterium. It propels *Shigella* through the cytoplasm until it hits the plasma membrane, and the contact force creates a protrusion into the neighbouring epithelial cell. Then, all the membranes are lysed by IpaB and IpaC which releases *Shigella* into the adjacent epithelial cell (Stevens *et al.*, 2006). At the same time, intracellular *Shigella* stimulates epithelial cells to release IL-8 together with IL-1β, which are released from apoptotic macrophages (chemotactic to PMN cells). This attracts and stimulates them to migrate to the lumen via the epithelial layer, which occurs as a result of the epithelial destruction the *Shigella* epithelial layer invasion amplifies (see Figure 1.5) (Jennison & Verma, 2004; Sani *et al.*, 2007; Singer & Sansonetti, 2004; Zurawski *et al.*, 2006).

All these processes aggravate bacterial infection and tissue lesion, resulting in diarrhoea and the pathological features that define shigellosis. In the end, however, the infection is brought under control when the bacteria are destroyed by the PMN mobilised to the infected area (Zhang *et al.*, 2001). Way *et al.* (1998) argued that the ability of an organism to withstand *S. flexneri* infection is to a great extent attributable to IFN-γ, which contributes to activating macrophages, thereby protecting them from bacteria-induced apoptosis. Nevertheless, uptake of water, nutrients, and solutes are significantly affected by the serious tissue damage arising from *Shigella* spp., resulting in the main manifestations of shigellosis (i.e. watery diarrhoea and blood and mucus in stool). Additional ramifications include impaired

electrolyte homeostasis and altered membrane transport processes (e.g. unregulated ion and fluid secretion) (Laohachai *et al.*, 2003).

Notably, recent studies have reported that, during the initial phases of infection, *S. flexneri* was associated with crypts, which include the cells that enable epithelial cells to regenerate, namely the stem cells of the colonic epithelium that can divide and differentiate into every type of cell present on the surface of the colon. Hence, invasion of this area by pathogenic agents could have major implications for the tissue. Moreover, in addition to the active infection, long-term complications can arise from the damage, which disrupts tissue repair or fosters the development of cancers. However, Arena *et al.* (2015) emphasised that indirect effects are the most probable determinants of long-term implications. This is because the stem cells are at the bottom of the crypts and are therefore largely inaccessible to *S. flexneri* for direct targeting.

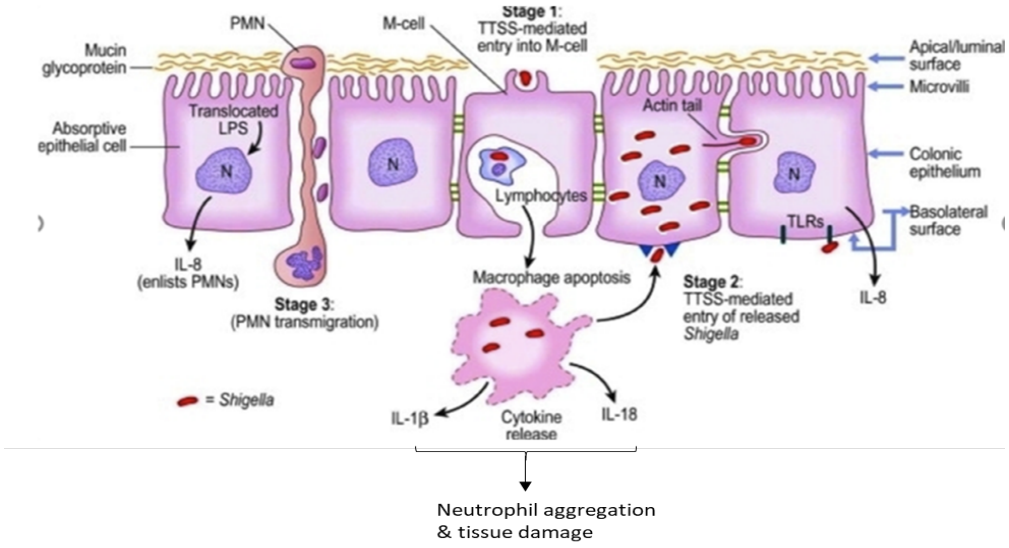


Figure 1. 5. Representation of the cellular pathogenesis of *Shigella* spp. *S. flexneri* using M cells to permeate the epithelial cell barrier via transcytosis, bacteria trigger apoptosis to escape macrophages, leading to pro-inflammatory signalling. Epithelial cells are accessed basolaterally by free bacteria, which enter the cytoplasm via vectorial actin polymerisation and diffuse to neighbouring cells. The innate immune response with the participation of NK

cells and PMN mobilisation is activated by pro-inflammatory signalling. Infection and tissue damage are aggravated at first by the PMN-based destruction of the epithelial cell lining which enables more bacteria to invade. The infection is eventually controlled when the PMN destroys the bacteria (Bliven & Maurelli, 2012).

1. 5. 3. Pathogenic features and Virulence mechanisms employed by AIEC

It remains to be established whether AIEC foster IBD by inducing intestinal inflammation or whether they institute mucosal colonisation of individuals with pre-existing inflammatory disease, thereby constituting an aggravating factor. AIEC persists in the gut mucosa, and inflammation is supported by several factors. Some authors have suggested that AIEC invasion of mucosal cells is aided by atypical mucosal immunity, intestinal barrier impairment, or other defects. This is attributed to the fact that pathogenicity genes have not yet been discerned (Ellinghaus *et al.*, 2012; Elliott *et al.*, 2013). Motility, capsule and LPS expression, serum resistance, iron assimilation, epithelial cell line attachment and invasion, and biofilm development are stimulated by AIEC virulence genes. Furthermore, these are regulated by bacterial components and regulatory pathways in AIEC strains. For instance, IEC interactions are underpinned by lipoproteins (Barnich *et al.*, 2004; Rolhion *et al.*, 2005), histone-like proteins (Miquel *et al.*, 2010), ribonucleotide reductase NrdR (Dreux *et al.*, 2015), flagellar transcription regulator FlhD2C2, sigma factor FliA, and the second messenger cyclic dimeric GMP (c-di-GMP) (Claret *et al.*, 2007). Contrastingly, biofilm development occurs with the sigma (E) pathway involvement (Chassaing *et al.*, 2015; Rolhion *et al.*, 2007). Furthermore, AIEC displays the pathogenic characteristics of biofilm formation on intestinal epithelial cells (IECs) and long polar fimbriae (LPF) (Chassaing *et al.*, 2011). AIEC can also survive and proliferate in macrophages owing to the stress protein HtrA (Bringer *et al.*, 2005), thiol-disulfide oxidoreductase DsbA (Bringer *et al.*, 2007), RNA-binding protein Hfq (Simonsen *et al.*, 2011) and FAD-dependent oxidoreductase IbeA (Cieza *et al.*, 2015). In each case, these support reactive oxygen species (ROS) survival. Moreover, Chassaing *et al.* (2011) reported

that translocation over M cells superimposing Peyer's patches was achieved by the AIEC strain LF82 via LPF.

LF82 attaches to the glycosylated receptor with hyperexpression in CD, referred to as CEACAM-6, and AIEC adhesion is enhanced by the receptor hyperexpression resulting from one of the following: firstly, AIEC invasion of epithelial cells; or secondly, pro-inflammatory cytokine promotion (Barnich *et al.*, 2007). Carvalho *et al.* (2009) confirmed this by revealing that AIEC triggered severe colitis in a humanised mouse model with CEACAM-6 hyperexpression. The CD-related localised stress reaction was identified as enabling AIEC invasion of epithelial cells, and the expression of the GP96 endoplasmic reticulum chaperone protein served as a receptor for protein OMP-A in the AIEC-secreted external membrane vesicles (Rolhion *et al.*, 2010). This is relevant because the X-box binding protein 1 (XBP1) encoded by the XBP1 gene in humans, which is a major transcription factor for cell stress reactions, contains an IBD-related genetic polymorphism (Kaser *et al.*, 2010).

Persistent colonisation is achieved through biofilm formation in LF82 and other serogroup O83 AIEC strains (Martinez-Medina *et al.*, 2009b). Furthermore, the risk of bacterial translocation to the submucosa and macrophage infection is heightened by AIEC-induced epithelial barrier dysfunction. This rearranges the tight junction adaptor protein zona occludens-1 (Wine *et al.*, 2009), as well as F-actin and E-cadherin from the apical junctional complex (Sasaki *et al.*, 2007). It is also worth emphasising that both dysbiosis and AIEC proliferation are promoted without host cell apoptosis by the AIEC infection-induced release of TNF-α (Bringer *et al.*, 2012; Glasser *et al.*, 2001; Lapaquette *et al.*, 2012), as well as additional pro-inflammatory cytokines (De la Fuente *et al.*, 2014). Additional AIEC properties include intestinal inflammation promotion by stimulating neutrophil ROS production (Vong *et al.*, 2016), antimicrobial response suppression by inhibiting interferon γ (IFN-γ)-mediated signal transducer and activator of transcription-1 phosphorylation in IEC (Ossa *et al.*, 2013), and promotion of production of pro-inflammatory cytokines by intestinal

epithelial cells, followed by translocation of leukocytes and dendritic cells (Eaves-Pyles *et al.*, 2008).

1. 6. Genes implicated in virulence and pathogenesis of *Shigella* and AIEC

1. 6. 1. Intra- and inter-cellular movement- IcsA (VirG) and mxiD

The genes *MxiD* and *icsA* are located in the ≈200-kb virulence plasmid of *Shigella sonnei* (*S. sonnei*). The mobility of *Shigella* in epithelial cell cytosol is made possible by the participation of *icsA* in actin-based motility (Bernardini *et al.*, 1989). Actin filament polymerisation and rearrangement on the surface of bacteria underpins the intra-inter cellular spread (ICS) movement. As a result, protrusions form and facilitate the passage of bacteria into neighbouring cells (Bernardini *et al.*, 1989; Kadurugamuwa *et al.*, 1991). Through the lysis of the cellular membranes surrounding the bacteria in the protrusions, the bacteria are released into the neighbouring cell cytoplasm, and therefore intercellular diffusion comes full circle (Allaoui *et al.*, 1993). It is notable that the secretion of *icsA* occurs close to the old pole of the *Shigella* cell, and the cell division protein FtsQ participates in the process underpinning intracellular modulation of this localisation (Fixen *et al.*, 2012). Notably, another function of *icsA* has been proposed recently in the literature, namely the involvement in polar adhesion of *Shigella* to epithelial cells (Brotcke Zumsteg *et al.*, 2014). The reliance of adhesion activity on the assembled T3SS rather than on the secretion of T3SS effectors was suggested by the fact that an ipaBCDA−mxiE− mutant was associated with icsA-mediated adhesion, whereas an ipaD−spa33− strain was not. Where it was found that the adhesion feature can be isolated from actin-based motility, as an icsA− mutant complemented by a plasmid encoding an adhesion-defective icsA formed plaques close to *Shigella* wild-type. In the Sereny test, the adhesion-defective *icsA* also developed an attenuated phenotype of infection, suggesting the significance of *IcsA* as an adhesive in *Shigella* pathogenesis. This initial finding was then linked to IcsA's T3SS-dependent activation to mediate this adhesive

phenotype, as the bile salt deoxycholate (DOC) application resulted in an increase in *IcsA*-dependent adhesion (Brotcke Zumsteg *et al.*, 2014).

The *Shigella* TTSS base structure consists mainly of the ring-forming proteins MxiD, MxiJ, and MxiG (Figure 1. 3) (Blocker *et al.*, 2001; Schuch & Maurelli, 2001; Tamano *et al.*, 2000). These proteins present cleavable N-terminal sec-dependent export signals, and they undergo treatment and translocation into the envelope when other types of III proteins are not present. Export is likely followed by the reaction of the MxiG and MxiJ rings with the MxiD periplasmic extension, which yields the basic transmembrane structure. The TTSS needle complex is achieved through cytoplasmic bulb protein export and nucleation of the needle extension subunits around and inside the envelope-spanning base (Schuch & Maurelli, 2001). The *mxiD* gene is found on the sizable virulence plasmid pWRIOO, upstream from (but orientated identically to) the *mxiA* and *spa* genes. Allaoui *et al.* (1993) reported that *mxiD* mutation made a new strain unable to penetrate HeLa cells, and also induced keratoconjunctivitis in guinea pigs. Additionally, most *S. flexneri* polypeptides, including IpaA, IpaB, and IpaC, were no longer secreted upon inspection of the *mxiD* mutant culture supernatant (Allaoui *et al.*, 1993).

1. 6. 2. *proQ.*

As a soluble ~25 kDa, a 232-residue protein with structural domains connected by an unstructured linker (Kunte *et al.*, 1999; Smith *et al.*, 2004), *proQ* was identified in *E. coli* based on its impact on osmolyte accumulation (Kunte *et al.*, 1999; Milner & Wood, 1989). The structural domains of *proQ* have been modelled in the literature, and structural predictions have been informed by biochemical research (Chaulk *et al.*, 2011; Ghetu *et al.*, 2000; Smith *et al.*, 2004). The findings reveal that *proQ* operates as an RNA chaperone. RNA-binding activity was first suggested when comparable features were observed in the protein sequence to FinO, where proteins with *proQ*/FinO domain(s) were identified in several proteobacteria (Smirnov *et al.*, 2016;

Smith *et al.*, 2004). Examinations of *proQ* in *E. coli* have revealed RNA-binding activity *in vitro* (Gonzalez *et al.*, 2017), while *proQ* locus disruption does not impact *proP* transcription and substantially decreases proline uptake activity in a Δ*proQ* strain (Kunte *et al.*, 1999; Milner & Wood, 1989).

ProQ works and interacts via base pairing with Small noncoding RNAs (ncRNAs) and target mRNAs (Smirnov *et al.*, 2017). In general, ncRNAs alter their target gene expression by direct base pairing with mRNA regions, resulting in a shift in translation efficiency or mRNA stability (Guo *et al.*, 2014). Small non-coding RNAs (ncRNAs) control gene expression in prokaryotes, regulate processes such as nutrient acquisition, stress response, virulence, and the formation of biofilms (Papenfort *et al.*, 2015; Wagner & Romby, 2015).

The lack of *proQ*- gene regulation in infectious bacteria has triggered upregulation of the metabolic system in the host whereas immune, calcium, and G - signalling host pathways are down-, with mitogen- protein kinase (MAPK) signalling being the most severely repressed host pathway (Westermann *et al.*, 2019).

Osmotic balance is key for bacterial survival. The importation of osmoprotectant molecules can balance osmolarity, both external and internal. It can also hinder the movement of water from the cell (Wood, 2006). Several membrane-bound transporters have been identified with diverse specificities for molecules. *ProP* detects hyperosmotic stress and imports glycine betaine and proline (MacMillan *et al.*, 1999; Wood, 1999). This promotes cellular hydration and facilitates bacterial growth in high-salinity media (Culham *et al.*, 2001; MacMillan *et al.*, 1999). In this context, *proQ* lesions inhibited *E. coli* growth in such a media and changed the morphological features of the cells. Additionally, impaired expression of downstream locus prc plays a suspected role in cell division and protein quality control (Beebe *et al.*, 2000; Hara *et al.*, 1991).

Bacterial expression of *proP* from chromosomes or plasmid-based PBAD promoters were associated with these effects, and plasmid-based *proQ* expression reversed

them (Chaulk *et al.*, 2011; Kunte *et al.*, 1999). An intricate network of growth-phase and osmolarity-dependent control was identified in proP's transcriptional regulation, where *proP* transcription can result from a distal (P1) or proximal (P2) promoter (Mellies *et al.*, 1995). In the latter case, transcription was identified as having improved by the nucleoid-associated factor Fis (Xu & Johnson, 1995) and the cyclic AMP receptor protein (CRP) (McLeod *et al.*, 2002). Fis and CRP binding was found to mitigate P1 promoter transcription (Xu & Johnson, 1997). Additionally, the P1 promoter's activation was identified as occurring after subculture in fresh media, and it reacted to changes in osmolarity (Landis *et al.*, 1999).

1. 6. 3. *dsbA*

Yu and Kroll (1999) reported that the formation of a disulphide bond in proteins secreted by Gram-negative bacteria was significantly promoted by the soluble periplasmic protein, *DsbA*, which serves as a protein-folding catalyst. This protein plays a central role in the biogenesis of toxins and multimeric structures on the bacterial surface. Disulphide-bonded proteins cannot develop in the cytoplasm of wild type *E. coli* owing to the occurrence of many reductases and reducing agents such as glutathione (Figure 1.6B). Evidence shows that glutathione is actively involved not only in apoptosis but also in the regulation of the functions of cells with antigen. Furthermore, as S-nitrosoglutathione, glutathione plays the role of a carrier molecule for nitric oxide (Venketaraman *et al.*, 2003), secretion of proteins that require disulphide bonds for their folding has to occur in the periplasm, which in *E. coli* is where disulphide bonds are formed and corrected through the involvement of a series of cell envelope proteins (Dsb), which act as catalysts (Kadokura & Beckwith, 2010) (Figure 1.6A).

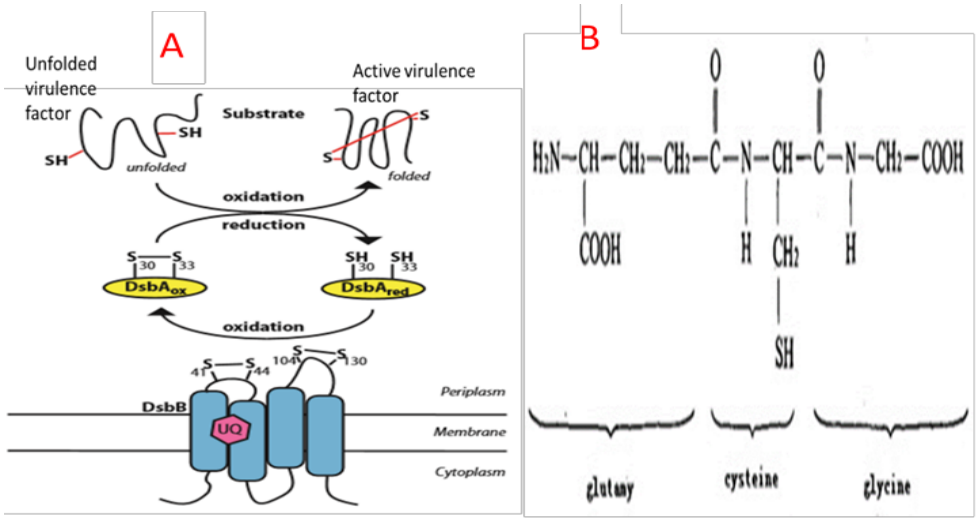


Figure 1. 6 AB. (A) Schematic representation of the Dsb cycle. (B) The chemical configuration of glutathione; DsbA and a substrate protein (P) form a mixed disulphide bond that results in oxidised (disulphide bonded) P and reduced DsbA; DsbB undertakes re-oxidation of DsbA and therefore could be a potential site for terpenoids with effect against Gram-negative bacteria, including *Shigella* adapted from (Halili *et al.*, 2015).

Ample research has been conducted to explore the features of the 21 kDa monomeric protein *dsbA*, both *in vivo* and *in vitro*. Consisting of a classic thioredoxin fold (Pan & Bardwell, 2006) and a CxxC active site motif, *dsbA* is an oxidase of great potency (Huber-Wunderlich & Glockshuber, 1998). It serves as a catalyst for disulphide bond formation and as a substrate protein accessing the periplasm (Kadokura *et al.*, 2004). *DsbA* undergoes reduction as a result of donating its disulphide bond to the substrate, and in order to revert to its active oxidised form, the inner membrane protein DsbB subjects it to re-oxidation (Messens & Collet, 2006). Similarly, electrons are donated by dsbB to ubiquinone (coenzyme Q) or menaquinone, depending on whether growth is aerobic or anaerobic, respectively (Bader *et al.*, 1999). Due to its great potency as an oxidase, *dsbA* can catalyse disulphide bond formation even in proteins in which

such bonds are typically absent. For instance, expression of the usually reduced beta-lactamase CcrA from *Bacteroides fragilis* in the *E. coli* periplasm makes the protein inactive, as its cysteines undergo oxidation (Elksne & Rasmussen, 1996). Likewise, dsbA oxidizes cytosolic proteins with typically reduced cysteines transported to the periplasm, and they are also misfolded. To enter the periplasm, recombinant proteins are fitted with an N-terminal signal sequence that directs them to the Sec translocon, the main protein-conducting channel in the cytoplasmic membrane that facilitates the biogenesis of both membrane and secretory proteins; where secretory proteins are translocated through the Sec translocon in a mostly unfolded state (Arkowitz *et al.*, 1993; du Plessis *et al.*, 2011).

Yu (1998) indicated that *dsbA* was crucial to the survival and growth of *S. flexneri* in the intracellular medium (Figure 1. 6 A). *DsbA* is also involved in the oxidative folding of Spa (Schuch *et al.*, 1999), which is an external membrane protein of the *Shigella* type III secretion system that underlies the generation of Ipa protein (Watarai *et al.*, 1995) (see figure 1. 4). According to Smith *et al.* (2016), bacteria stopped spreading to neighbouring cells and virulence was suppressed as a result of *dsbA* knockout mutant Sh4. In the literature, the LF82-*dsbA* mutant has not been observed to express flagella or type I pili; Where reported previously, in *E. Coli, DsbA* participates in the post-translation modification of a flagellar synthesis protein by catalysing the formation of disulfide bonds in the P-ring protein FlgI (Dailey & Berg, 1993). This lack of expression seems to be the sole input of *dsbA* in the adherence, as the forced interaction between bacterium and cell was necessary for the recovery of the wild type phenotype (Bringer *et al.*, 2007). At the same time, the expression of flagella and type I pili was needed to induce it.

The *dsbA* gene is vital for the ability of AIEC LF82 to survive in macrophages. This ability has been identified as having similarities to that of harmless *E. coli* K-12, which is effectively eliminated by macrophages. In addition, *dsbA* is crucial for resistance to eradication in the acidic macrophage phagolysosome (Bringer *et al.*, 2007).

1. 7. Antimicrobial resistance and alternative therapy

As bacteria are becoming increasingly resistant to a growing number of drugs, the existing medication-based treatment choices are diminishing in terms of their efficiency. Under such circumstances, researchers have started to pay attention to drugs and compounds such as terpenoids, which can be employed not to destroy bacteria but to deactivate them. This would play a valuable role in helping to solve the twin issues of bacterial resistance and the elimination of beneficial gut bacteria.

Terpenes, or terpenoids, are organic compounds that target bacteria (Mendoza *et al.*, 1997) as well as fungi (Rana *et al.*, 1997) and viruses (Sun *et al.*, 1996). Monoterpenes are present in the essential oils extracted from various plants, and these compounds are the source of the aroma and flavour of the plants. Alongside the substrate geranyl pyrophosphate (GPP), monoterpenes are synthesised by monoterpene synthases. There are three types of monoterpenes: acyclic, monocyclic, and bicyclic C30 compounds. Cyclic monoterpenes are the products of monoterpene cyclases, which employ a terpinyl cation amenable to conversion into multiple compounds. Monoterpenes are responsible for activating the expression of phase-I and phase-II hepatic detoxification enzymes; selectively inhibiting protein isoprenylation; triggering the mannose G phosphate/IGF-II receptor, and inducing expression of TGF-β. Furthermore, there is evidence that some monoterpenes exhibit anti-cancer effects at various cellular and molecular levels. The essential oil derived from lemongrass (Cymbopogon) and additional aromatic herbs contain the monoterpene geraniol. Several studies that have analysed the effects of geraniol on multiple cell lines, both *in vivo* and *in vitro*, indicate that the compound can successfully target cancerous cells (Burke *et al.*, 2002; Cho *et al.*, 2016; Crowell, 1999). Moreover, as reported by Lorenzi *et al.* (2009), geraniol targeted the virulence factors of pathogenic Gram-negative bacteria like efflux pump inhibitors (EPIs), including isolates with resistance to multiple drugs (e.g., *Enterobacter aerogenes*, *E. coli*, and *P. aeruginosa*).

The fact that an increasing number of bacteria are acquiring resistance to antibiotics makes the treatment of bacterial infections increasingly challenging. Nevertheless, a novel drug for treating bacterial infections, and particularly urinary tract infections, has been developed by researchers at the University of Brussels. Unlike standard antibiotics, this drug does not destroy bacteria but deactivates them (Lo *et al.*, 2014).

The main use of essential oils (EOs) is as flavourings in the food industry, but they are also a noteworthy source of natural antimicrobials (Hyldgaard *et al.*, 2012). The manner in which the antimicrobial effect of terpenes is exerted must be understood in order to exploit it effectively. As emphasised by Carson and Riley (1995), there is more than one mechanism of EO antimicrobial action, and multiple sites in a cell are probably targeted. Furthermore, not all strains are equally susceptible to a particular EO, and thus the prediction of organism sensitivity is challenging. Nonetheless, Gram-positive bacteria are generally considered to have higher sensitivity than Gram-negative bacteria (Trombetta *et al.*, 2005). The latter are less sensitive due to the lipopolysaccharides (LPS) present in their external membrane, which hinders the access of macromolecules and hydrophobic compounds. Consequently, EO antimicrobial compounds, which are hydrophobic, are tolerated more effectively in Gram-negative bacteria (Nikaido, 2003). EOs take advantage of this hydrophobicity to increase the permeability of the cell membranes of Gram-positive bacteria and, to a lesser degree, Gram-negative bacteria, which occurs through the disruption of the lipids they contain (Sikkema *et al.*, 1994). In turn, this leads to the loss of cellular material and ions (Carson *et al.*, 2002). The extent of that loss determines whether the cells die or not (Chovanov¨ç *et al.*, 2013; Johnston *et al.*, 2003).

Most terpenoids derive their antimicrobial action from the functional groups they contain. For instance, Hyldgaard *et al.* (2012) reported that the antimicrobial action of phenolic terpenoids was related to the existent delocalised electrons resembling reduced glutathione and a hydroxyl group. Carvacrol and thymol induced the release of lipopolysaccharides and made the cytoplasmic membrane more permeable to ATP, specifically by breaking down the external cell membrane of Gram-negative bacteria.

Ultee *et al.* (2002) reported that carvacrol enhanced membrane permeability by separating the fatty acid chains in the phospholipids, thereby creating channels in the membrane.

1. 8. Biofilm and models for studying the virulence and pathogenesis of *Shigella* and AIEC

1. 8. 1. biofilm formation

Bacterial biofilms are clusters of a single or multiple bacterial species enclosed in a slimy matrix comprising polysaccharides, DNA, and proteins. Bacterial biofilms are also capable of attaching to different surfaces (Richards and Melander, 2009). In fact, there is no surface that biofilms cannot thrive on, including lake bottoms, medical implants, and tooth film coating. These are frequently visible macroscopically (Costerton *et al.*, 1999; Khatoon *et al.*, 2018). The main stages and mechanisms of biofilm formation are attachment to the surface (initial adhesion, permanent adhesion), microcolony (early biofilm development), biofilm maturation and detachment (spread of individual cells from the biofilm) (Crouzet *et al.*, 2014; Stoodley *et al.*, 2002).

Bacterial infections associated with biofilms have been identified as more than one thousand times more resistant to antibiotics and environmental stress than free-floating or planktonic forms of bacteria. This explains the great interest given to biofilms in disease pathogenesis research (Parsek & Singh, 2003). Hall-Stoodley and Stoodley (2005) argued that the microbial biofilm state was a likely reason why bacterial biofilms persisted on medical implants or damaged tissue. Antibiotic resistance and an ability to escape the innate immune response in bacterial biofilms may contribute significantly to the development of conditions such as chronic lung infection, cystic fibrosis, and infections related to the insertion of central venous catheters (Hall-Stoodley & Stoodley, 2005; Ramos *et al.*, 2010).

The occurrence of biofilms and microbial infections with chronic persistence has been accounted for using several theories. Disease symptoms can reappear following repeated antibiotic regimens because, despite the fact that antibiotics can kill biofilm-released free-floating planktonic cells, they cannot eradicate the biofilm itself. Therefore, the colonised biofilm surface must be excised to ensure the effectiveness of the treatment (Stewart & Costerton, 2001). The literature indicates that environmental stressors, including scarcity of nutrients, pH variability, disinfectants, and antibiotics, have no effect on biofilms (Jefferson, 2004; Singh *et al.*, 2017). Biofilms defend themselves by exploiting the capacity of the matrix to retard antibiotic dissemination via the different polymeric substances they contain (Gebreyohannes *et al.*, 2019; Stewart & Costerton, 2001).

Bacterial resistance to antibiotics has also been explained in terms of the fact that, owing to nutrient scarcity, the bacteria in biofilms exist in a semi-starved condition that retards their growth. In turn, this passivity reduces their sensitivity to antibiotics (Roy *et al.*, 2018; Taraszkiewicz *et al.*, 2013). Furthermore, as described by Costerton *et al.* (1999), there is a nutrient and waste gradient in biofilms, whereby resources are more accessible to bacteria on the biofilm surface than to those at a greater depth in the biofilm. In this context, the former generates a higher amount of metabolic by-products and the latter are associated with higher amounts of waste products. Therefore, the bacteria within the biofilm depth enter a non-growing state and enjoy protection against antibacterial effects. This stems from the way in which certain antibiotics (e.g. penicillin) only have the capacity to destroy actively growing bacteria (i.e. because they target cell-wall synthesis) (Costerton *et al.*, 1999).

1. 8. 2. *Galleria* moth larvae model

A suitable animal model is often needed to investigate bacterial virulence. However, mammalian models of infection are costly and require ethical approval (Tsai *et al.*, 2016). The use of insects as infection models provides a valuable alternative. Compared to other non-vertebrate model hosts such as nematodes, insects have a relatively advanced system of antimicrobial defences. Due to this, they are more likely to produce information relevant to the mammalian infection process. Like mammals, insects possess a complex innate immune system (Lemaitre & Hoffmann, 2007). Cells in the haemolymph, which is analogous to mammalian blood, contain immune cells called haemocytes, which can be compared to mammalian neutrophils in terms of their ability to phagocytose and kill pathogens through superoxide production (Bergin *et al.*, 2005; Renwick *et al.*, 2007), as well as humoral responses, which include the inducible production of lysozyme and small antibacterial peptides such as complement-like proteins (opsonins), melanin, and antimicrobial peptides (AMPs) (Tsai *et al.*, 2016; Vodovar *et al.*, 2004). In addition, the epithelial cells of insect larval midguts and intestinal cells of mammalian digestive systems are similar in certain respects. It is also noteworthy that several basic components, each of which is essential for the bacterial infection process (e.g. cell adhesion, resistance to antimicrobial peptides, tissue degradation, and adaptation to oxidative stress), are likely to be important in both insects and mammals (Lemaitre & Hoffmann, 2007). For this reason, insect models can serve as polyvalent tools for the identification and characterisation of the microbial virulence factors associated with mammalian infections.

Early studies that used *Galleria mellonella* (*G. mellonella*) as an infection model for phage therapy research showed promising outcomes. Phages were identified as a way to cure bacterial infections effectively in Nale *et al.* (2016). The researchers focused on the therapeutic characterisation of isolated bacteriophage against *E. coli*,

and they evaluated the stability of freeze-dried phages for long-term storage. The researchers also assessed the efficacy of bacteriophage using a *G. mellonella* model.

Larvae of the greater wax moth, *G. mellonella* (also known as the honeycomb moth), have provided useful insights into the pathogenesis of a wide range of microbial infections, including mammalian fungal pathogens (e.g. *Fusarium oxysporum*, *Aspergillus fumigatus*, and *Candida albicans*) and bacterial pathogens (e.g. *Staphylococcus aureus*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Enterococcus faecalis*) (Jander *et al.*, 2000). Regardless of the bacterial species, results obtained with larvae of the greater wax moth, infected by direct injection through the cuticle, correlate consistently with those of similar mammalian studies. Bacterial strains that are attenuated in mammalian models demonstrate lower virulence in *G. mellonella*, and strains causing severe human infections are also highly virulent in the *G. mellonella* model (Cadot *et al.*, 2010; Gao *et al.*, 2010). However, oral infection of *G. mellonella* is much less used, and additional compounds (e.g. specific toxins) are needed to induce mortality. Infection of the larvae enables the monitoring of bacterial virulence by several means, including calculation of Lethal Dose (LD50) (Finney, 1952), measurement of bacterial survival (Fedhila *et al.*, 2002; Guillemet *et al.*, 2010), and examination of the infection process (Nielsen-LeRoux *et al.*, 2012).

1. 8. 3. Organoids

1. 8. 3. 1. Gut Development

The digestive tract of vertebrates is essentially a primitive tube stretching from the mouth to the anus, and it consists of several specialised regions. The gut is developed via endodermal layer folding, and it can be separated into the foregut, the midgut, and the hindgut (Lewis & Tam, 2006). Specific organs are formed at each of these parts, and these emerge at different stages of the pre-natal period. Thus, the foregut gives rise to the pharynx, oesophagus, stomach, liver, pancreas, and lungs, as well as a portion of the duodenum. The midgut is responsible for the formation of what remains of the duodenum, along with the jejunum, the ileum, and portions of the large intestine. Finally, the hindgut underpins the formation of the rest of the large intestine (Lewis & Tam, 2006).

The basal crypt, which maintains and rejuvenates the epithelium, and the villus domain, which ensures that nutrients are assimilated from the gut lumen, are the two locations towards which the single layer of epithelial cells lining the intestine can be guided (van der Flier & Clevers, 2009). Intestinal stem cells (ISCs, from which every type of intestinal epithelial cell is formed), Paneth cells, and transit-amplifying (TA) cells are the three main categories of cells that are observed in the crypt domain. The Lgr5 transmembrane protein, which participates in the Wnt signalling pathway, is expressed by ISCs. Furthermore, peptides that target microbes, which protect the function of stem cells, are produced by Paneth cells. Therefore, they play a role in the innate immune system. The types of cells present in the villus are generated by TA cells, and these exhibits partial differentiation. Meanwhile, enteroendocrine cells, enterocytes, and goblet cells are the major classes of cells that reside in the villus domain. Regulation of nutrient metabolism is undertaken by the enteroendocrine cells through the production of hormones, whilst nutrient absorption occurs with the involvement of enterocytes. Protection of the epithelium and facilitation of the

movement of food through the intestinal lumen are ensured by the goblet cells, specifically via their production of mucous.

1.8.3.2. Small intestine

The small intestine is a tube-shaped organ, 1 inch in diameter and around 12 feet long. The small intestine starts from the pyloric sphincter of the stomach and ends in the ileocecal valve, which is the starting point of the large intestine. According to the function and structure of the small intestine, three parts can be distinguished (Figure 1. 7 A). The first part starts at the pyloric sphincter and is known as the duodenum. Measuring 10 inches, the duodenum consists of four portions, namely the superior, descending, horizontal, and ascending duodenum. It becomes retroperitoneal when it angles to the posterior at the rear of the peritoneum, immediately after the pyloric sphincter, subsequently surrounding the head of the pancreas in a C-shape and then going upward anteriorly, returning to the peritoneal cavity and connecting with the jejunum. The jejunum is the second part of the small intestine, spanning a length of around 3 feet between the duodenum and the ileum. It is structurally almost the same as the ileum, apart from the expanded lumen and the greater multiplicity of internal folds. The ileum is the last part of the small intestine, measuring 6-7 feet. The ileum wall lining is made up of lymph nodules called mesenteric patches or Peyer's patches. The ileum ends in the medial part of the cecum (Parrish & DiBaise, 2017).

The external serosa, muscularis, submucosa, and internal mucosa comprise the four layers of the small intestine (Figure 1. 7 B). The epithelial lining of the mucosa is responsible for the assimilation of nutrients released from food broken down at the molecular level. Such assimilation occurs in every part of the small intestine, but especially in the jejunum. To ensure that the maximum amount of nutrients is assimilated, the mucosa is organised into villi, at the bottom of which are cavities known as intestinal glands (or Lieberkühn's glands). These glands are lined with cells that spread across the surface of the villi. At their base, they contain Paneth cells, which are a type of epithelial cell. The latter consist of alpha or eosinophilic granules,

which derive their name from the fact that they assimilate the rose-coloured stain eosin (Figure 1. 7 C) (Gerard J. Tortora 2014).

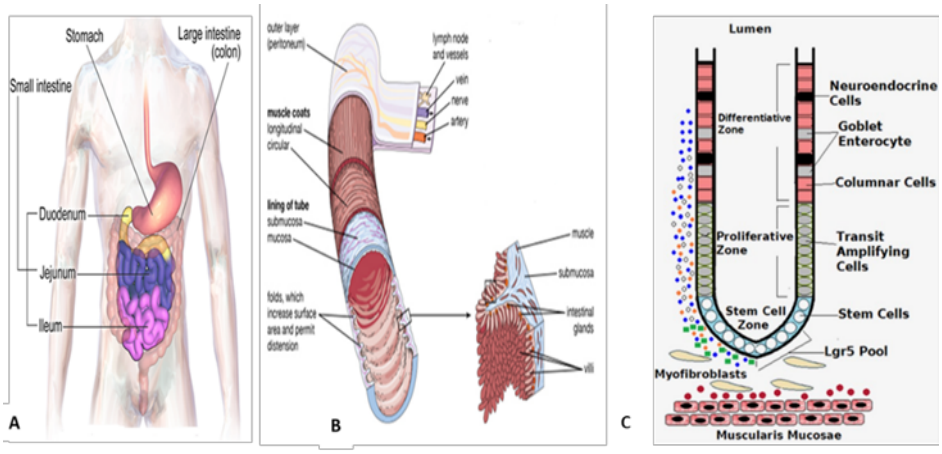


Figure 1. 7. (A) The structure of the digestive tract. (B) The walls of the small intestine cavity; the serosa is the external layer, smooth muscle layers, submucosa; the mucosa represents the internal layer lining the tract lumen. (C) The small intestine is composed of villus and crypt structures. LGR5þ stem cells and Paneth cells are located at the base of the crypts, followed by the TA cells, and then mature epithelium composed of goblet cells, enteroendocrine cells, and enterocyte adapted from (Fair *et al.*, 2018; Mahler *et al.*, 2009; William Sircus *et al.*, 2016).

1.8.3.3. Wnt signalling

The Wnts are glycoproteins that are secreted and consist of a large family of nineteen proteins in humans that suggest a daunting complexity of regulation, function, and biological output signals (Komiya & Habas, 2008).

The name of this process derives from the terms for the Drosophila segment polarity gene wingless and vertebrate homolog integrated (or int-1) (Bejsovec, 2018; Wodarz & Nusse, 1998). The Wnt signalling pathway represents a highly conserved signal transduction cascade (Figure 1. 8), which plays a crucial role in various biological processes (e.g. embryonic development and tissue regeneration). Gene expression

and cell differentiation depend on the Wnt concentration gradient, due to the concentration-based response of target cells to secrete Wnt morphogens. Consequently, the signal transduction pathways help cells to proliferate, survive, and differentiate, relying on Wnt molecules (Hobmayer *et al.*, 2000; Mills *et al.*, 2017; Peifer & Polakis, 2000).

The Wnt signalling pathway's functions in the gastrointestinal system include maintenance of the concentration of undifferentiated intestinal progenitor cells, regulation of differentiated Paneth cell maturation, and localisation. In addition to Paneth cells, stromal cells encircling the crypt and other ISC niche cells also produce Wnt ligands like Wnt3 (Kabiri *et al.*, 2014; Sato *et al.*, 2011b). Wnt signalling is enhanced by the Wnt agonist R-spondin. This occurs through the LGR-dependent mechanism, namely when Wnt ligands are present (de Lau *et al.*, 2014). Wnt and R-spondin ligands carry out different functions under ISC homeostasis. Wnt proteins require R-spondin ligands to trigger ISC self-regeneration and expansion *in vivo*, despite providing basal competency by upholding R-spondin receptor expression. The Lgr5+ISC pool size is regulated by R-spondin instead of Wnt (Yan *et al.*, 2017), and crypt proliferation and ISC maintenance are the functions of the R-spondin proteins produced by the intestinal stromal niche (Kabiri *et al.*, 2014; Kim *et al.*, 2005). However, both Wnt and R-spondin are important for ISC maintenance, since Wnt activity and ISC proliferation ceased and peri-cryptal mesenchymal cells with Foxl1 expression were depleted when Wnt ligands and R-spondin were lost (Aoki *et al.*, 2016).

Intestinal organoids are helpful in gaining detailed insights into the reactions that bacterial invasions elicit from the intestinal epithelium and, in particular, from ISCs. They are also useful when attempting to understand the interactions that occur between the intestine and intestinal microbiota under normal conditions. Furthermore, enteric, pathogen-caused intestinal dyshomeostasis can also be examined with intestinal organoids, as can microbe pathogenesis (Hill & Spence, 2017). The use of intestinal organoids to investigate how microbes become

pathogenic could contribute to the development of more effective treatments for various diseases, including CD. These new treatments could rely on antibiotics and alternative agents with antimicrobial activity (Yin & Zhou, 2018), including terpenoids.

Intestinal organoids are compatible with both *in vivo* and *in vitro* methods. As a result, they may catalyse the development of a more holistic strand of research. The adequacy of intestinal organoids as three-dimensional multicellular models stems from the fact that they have all the intestinal components that are crucial for adult mammal physiology (e.g., the major types of intestinal cells, a functional lumen, as well as crypts and villi). Another strength of organoids is that they can be genetically manipulated through DNA or siRNA transfection (Kraiczy & Zilbauer, 2019). It is also possible to develop organoid subcultures, and these can be expanded, frozen, or exposed to infectious pathogens. In two studies, the researchers reported that organoids from mouse intestinal crypts exhibited gene expression due to transfection or viral infection (Koo *et al.*, 2013; Koo *et al.*, 2011). Furthermore, organoids have been found to grow quickly in culture, and their ISCs can divide and differentiate rapidly. This is why organoids can be subjected to many different analytical techniques, including immunohistochemistry, mass spectrometry, microarrays, and sequencing (Leushacke & Barker, 2014). Additionally, as previously mentioned, intestinal organoids may be useful models for the investigation of intestinal disorders, including CD, coeliac disease, and ulcerative colitis.

Colorimetric assays and microscopy were applied by Grabinger *et al.* (2014) to intestinal organoids to examine epithelial apoptosis pathways. Additionally, Wilson *et al.* (2015) demonstrated that the targeting of microbes by Paneth cells could be observed through the introduction of a bacterial pathogen into an organoid lumen. Mouse models of intestinal organoids have been used to investigate how the organoid structure can be invaded by *Salmonella*, and what changes the structure underwent as a result. The results indicate that, in addition to disrupting epithelial tight junctions, *Salmonella* infection reduced leucine-rich-repeat containing G-

protein-coupled receptor 5 (Lgr5) and Polycomb complex protein (Bmi1) stem cell markers in immunofluorescent-labelled Lgr5 organoids. *In vivo* research employing organoids further revealed that *Salmonella* infection also induced modifications in the inflammatory response. Such results provide strong evidence in support of the use of cultured organoids to explore host-bacteria interactions (Zhang *et al.*, 2014).

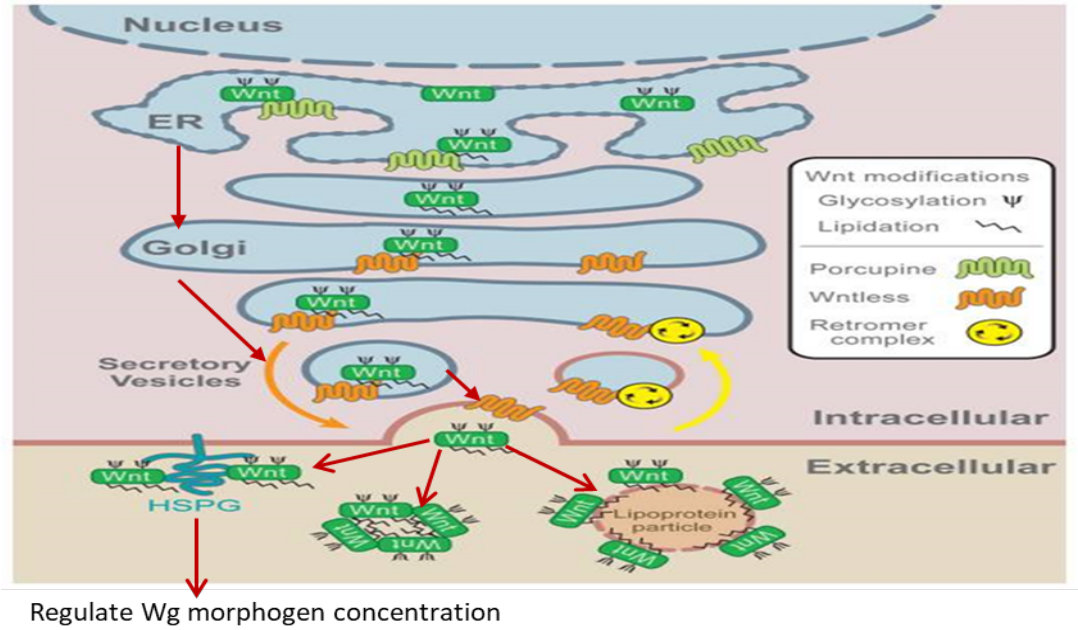


Figure 1. 8. Biogenesis and secretion of Wnt. Wnts are glycosylated and lipid-modified in the Porcupine-involved endoplasmic reticulum (ER) and secreted by Wntless from the Golgi to the plasma membrane. The retromer complex removes Wntless from endocytic vesicles back to the Golgi. Upon mature secretion, Wnts bind to or form multimers of heparan sulfate proteoglycans (HSPGs) and lipoprotein particles, which can modulate Wnt gradients and promote long-range Wnt signalling. The number of Wnt molecules linked to the particle of lipoprotein and were arbitrarily drawn in the multimeric form. HSPGs play an important role in the Wg morphogen concentration through the control of Wg degradation, diffusion, endocytosis/transcytosis and may act as potential low-affinity co-receptors in Wg signalling (Lin, 2004), adopted from (MacDonald *et al.*, 2009).

1.8.3.4. Intestinal organoids

Intestinal organoids can be obtained from stem cells in an intestinal crypt (Onozato *et al.*, 2018) (Figure 1. 9). An organoid accurately replicates an organ and gland structure at the micro-level (Serra *et al.*, 2019; Shamir & Ewald, 2014). This is because the organoid is made up of glandular and organ elements instead of a single type of tissue or cell (Figure 1. 10).

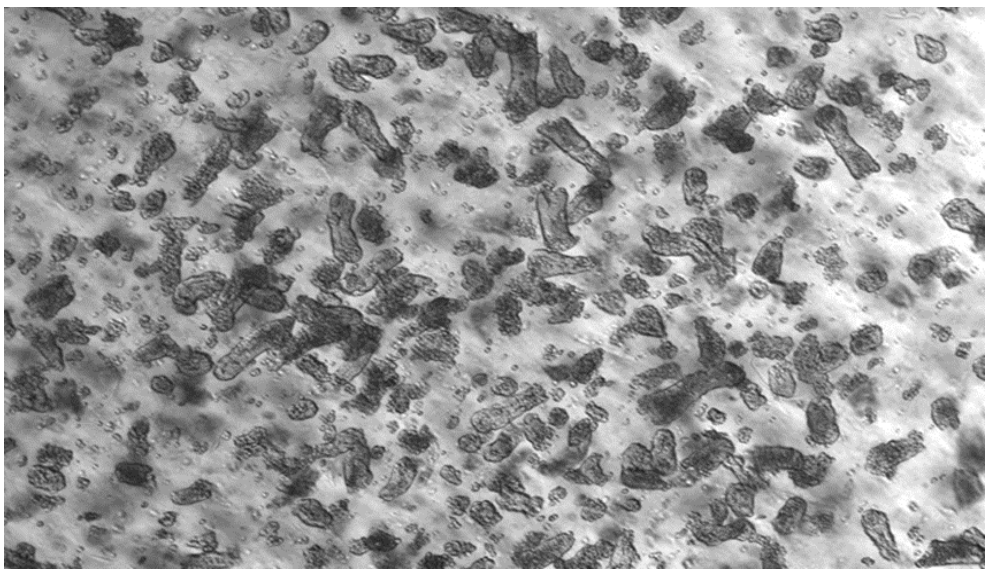


Figure 1. 9. A bright-field image of a 10 µl aliquot from a 200 µl resuspension of colon crypts. Scale bar = 50 µm, adopted from (O'Rourke *et al.*, 2016).

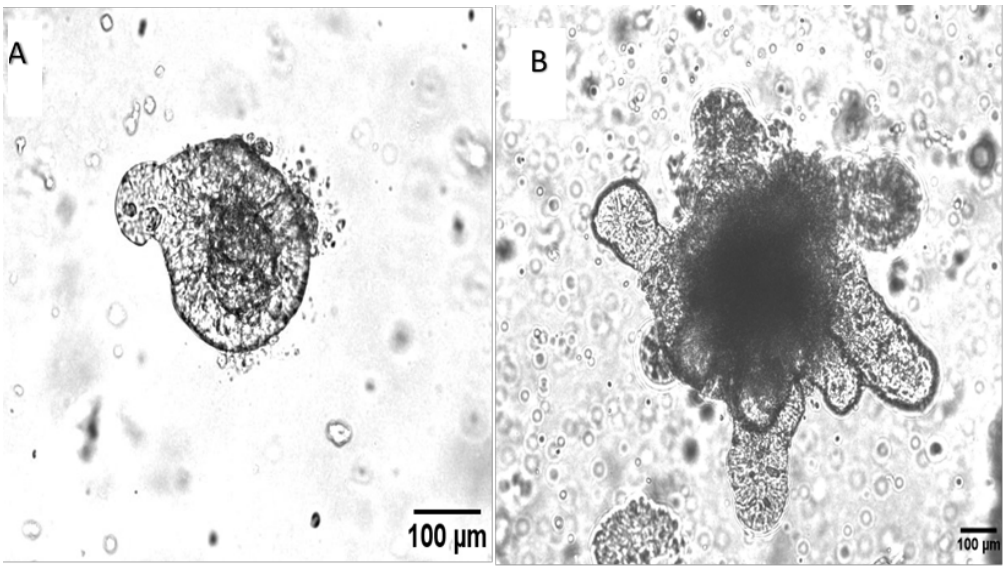


Figure 1. 10. Organoids created from mouse intestinal crypts under conditions of 37°C temperature and 5% carbon dioxide, shown at two (A) and seven days (B). The epi-fluorescent inverted microscope (scale bar = 100 µm) was used for visualisation, while a bright-field microscope was employed to regularly monitor the chosen crypts (organoids) per well after plating of the crypts. The organoids began to grow two days after the creation of the culture. The emerging buds constituted crypt structures and differed in the number between separate organoids. Shed epithelial cells started to aggregate in the organoid lumen on the fifth day, possibly due to epithelial turnover, which has a cycle of about five days (Sato *et al.*, 2009).

1.8.3.5. Intestinal renewal

Intensive renewal is made possible by the two key attributes exhibited by intestinal stem cells (ISCs), namely longevity and multipotency. Once multiplication ceases, ISCs undergo differentiation into the intestinal epithelium (Evans *et al.*, 1992; Nakamura & Sato, 2018). The creation of a three-dimensional organoid from mouse intestinal crypts was first achieved by the Clevers group (Sato *et al.*, 2011a; Sato *et al.*, 2011b; Sato *et al.*, 2009). Meanwhile, (Hughes *et al.*, 2010a) noted that extracellular matrix, which is usually Matrigel consisting of extracellular proteins (e.g. laminin, enactin, and collagen IV), was conducive to the growth of organoid cultures. Hence, Insulin-producing cells (IPC) culturing can be undertaken without myofibroblasts, while isolated cells that are extrinsic to normal tissue undergo detachment-induced apoptosis (referred to as anoikis) (Hofmann *et al.*, 2007). A Matrigel growth medium with abundant laminin enabled the use of ISCs to generate crypt-villus structures, as well as the major types of intestinal cells, including epithelial and mesenchymal cells. Moreover, for organoids to grow properly, epidermal growth factor (EGF), Wnt agonist (R-spondin-1), and Noggin (Bmp antagonist) are necessary.

The mouse submaxillary gland contains a peptide known as EGF. In humans, this peptide is found in the submaxillary gland and parotid gland, as well as in urine. In the latter case, it is referred to as urogastrone (Hollenberg & Gregory, 1980). The main function of the EGF is to help cells to grow, replicate, and differentiate. R-spondin-1, a protein, is expressed by the human Rspo1 gene, which is located on chromosome 1. It is responsible for activating Wnt4 and working in collaboration with it to stimulate the proliferation of cells. The increase in the number of crypts is promoted by the protein Noggin, which – in humans – is encoded by the NOG gene (Sato *et al.*, 2011a). During organoid development, the intestinal crypts are known as buds, and they are located near the central lumen. The presence of dividing LGR5-positive ISCs among Paneth cells indicates that the function of the buds does not differ considerably from that of mature intestinal crypts. Furthermore, the final

differentiation of ISCs into enterocytes, enteroendocrine cells, or goblet cells, followed by dissemination into the lumen, determines the formation of a villus-like area.

When fibroblast growth factor 4 (FGF4) and Wnt3a are present, monolayers of human embryonic stem cells (hESC), human-induced pluripotent stem cells (hiPSC), and endoderm, (the latter forming as a result of activin A stimulation) aid the development of hindgut spheroids. FGF4 and Wnt3a are both necessary for hindgut specification, but FGF4 activates hindgut morphogenesis alone. Further culturing of the spheroids leads to the formation of organoids with a polarised, columnar epithelium, exhibiting structures resembling villi and crypts (e.g. SCs expressing Lgr5, enterocytes, enteroendocrine cells, goblet cells, and Paneth cells). Following this, the organoids give rise to fibroblasts, intestinal subepithelial myofibroblasts, a mesenchymal layer, and smooth muscle cells (Spence *et al.*, 2011).

1.9. The aim of the study

Adherent-invasive *Escherichia coli* (AIEC) and *Shigella sonnei* are both involved in gastrointestinal diseases, where AIEC was found to have been implicated in Crohn's disease, whereas *Shigella sonnei* caused shigellosis. Both micro-organisms have shared inflammatory features and antibiotic resistance. For this reason, this study sought to compare the impacts of mutated genes in the same bacteria background, that implicated in the virulence and pathogenesis of *Shigella* and AICE such as *dsbA, yadA, proQ, icsA,* and *mxiD* using biofilm formation, *Galleria* models, raw macrophage, and intestinal organoids infection. Also, to assess and evaluate the antimicrobial activities of geraniol using the above bacteria, models, and techniques.

Chapter Two. Material and methods

2. 1. General Materials

There are different chemicals, and stains that were used in the present study, and their sources are presented in tables 2. 1.

Table 2. 1. Chemicals used in this study

Chemical Name	Source
Phosphate Buffer Saline (PBS)	Sigma Aldrich, UK
Dimethyl sulfoxide (DMSO)	
Ethanol (99.5)	
Ethidium bromide (EtBr)	
Foetal Bovine Serum (FBS)	
Congo Red	
Glycerol 99%	
Isopropyl-β-D-thiogalactopyranoside (IPTG)	
Luria Bertani (LB) with agar (Lennox)	
Luria broth base, Miller	
Non-essential amino acid	
Paraformaldehyde (PFD)	
Magnesium chloride	
Magnesium sulfate	
Sodium chloride	
Sorbitol	
Sucrose	
Arabinose	
Agarose	

Triton 100X	
Potassium di-hydrogen phosphate	
Glacial acetic acid	
Ethylenediamine Tetra acetic acid (EDTA)	
Sodium nitrite	
N-1 N-naphthyl-ethylenediamine dihydrochloride (NED)	
Sulphanilamide	
phosphoric acid (H_3PO_4)	
Ethylenediamine tetra acetic acid (EDTA) di-sodium di-hydrate	
Thiazolyl Blue Tetrazolium Bromide (MTT	
Sodium phosphate dibasic dihydrate	Fluka Analytical, Sigma Aldrich Co., UK
Trypan blue solution	Fluka, Sigma Aldrich, UK
Mueller Hinton Broth 2	Fluka Analytical, Sigma Aldrich Co., UK
Sodium dodecyl sulfate (SDS)	Fluka analytical. Sigma Aldrich Co., UK
Trise base	Fisher Scientific, UK
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	Promega, UK
Crystal Violet stain	PRO LAB DIAGNOSTICS IVD, UK
Alexa Fluor 546 Phalloidin	Molecular probes, USA

2. 2. Media composition, Antibiotics, and other supplements used in the present work

Antibiotics, chemicals, and some stains concentrations used in this study, all antibiotics and some stains were stored at -20°C.

Table 2. 2. composition of media used in the present work

Media	**Amount per Litre**
Lysogeny broth (LB) (Sambrook, 2001)	Lysogeny broth was prepared by adding 1% (w/v) of tryptone with 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl and adjusted to 1000ml of distilled water. pH adjusted at 7.0 with NaOH.
Super Optimal broth with Catabolite repression (SOC) (Hanahan, 1983)	Tryptone 2%(w/v), yeast extract 0.5% w/v), NaCl 0.05% (w/v), KCl 0.018% (w/v), added up to 1litre of distilled water, after autoclaving add sterilized components by filtrations with a 0.22µm filter: $MgSO_4.7H_2O$ 0.48% (w/v), Dextrose 0.36% (w/v).

Table 2. 3. DMEM media prepared for macrophage culture as follows

Ingredient	**Stock**	**Working solution**	**Preparations**
Sodium Pyruvate 1M (Sigma Aldrich, UK)	(100mg/ml) 100mM.	1mM	These elements were combined with a

Nonessential amino acids	100X	1X	DMEM (500ml) and passed through a filter sterilizer of 0.22 µm. Lastly, the mixture was refrigerated until use.
Penicillin/streptomycin (Sigma Aldrich, UK)	10000 unit and 10mg per ml	100U/0.1mg	
Fetal bovine serum (Sigma Aldrich, UK)		10%	

Table 2. 4. Buffers for isolating crypts

Buffer	**Amount per Litre**
Chelating Buffer	2ml of 0.5M Ethylene diamine tetraacetic acid (EDTA) dissolved in 498ml of 1XPBS. The pH was modified to 7.3. Then autoclaved and stored at 4°C (Mahe *et al.*, 2013)
Shaking Buffer	14.82 g/L of 43.3mM of sucrose and 10.82g/L of 54.9mM of Sorbitol to (1000ml) of 1XPBS, the pH was adjusted to 7.2-7.4. Then autoclaved and stored at 4°C (Mahe *et al.*, 2013)
Phosphate Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (Sigma Aldrich, UK)	

Table 2. 5. Mini-gut medium and their components with concentrations

It was prepared according to Mahe *et al.* (2013) as the following:

Ingredient	**Stock solution**	**Final Conc.**	**Volume 100ml**	**Function**
Advanced DMEM*/F12			94ml	Cell culture media
GlutaMax 0.2M (Gibco™) ThermoFisher sceintefic	100X	2mM	1ml	Prevent degradation and ammonia build up even long-term culture
4-(2-Hydroxyethyl) piperazine-1-ethane sulfonic acid, N-(2-Hydroxyethyl) piperazine- N'-(2-ethane sulfonic acid) (HEPES) 1M	100X	10mM	1ml	zwitterionic biological buffer and is one of Good's buffers, it is a more effective buffering agent for maintaining enzyme structure and function at low temperatures.
Penicillin 1000U/ml; streptomycin 100mg/ml (Sigma Aldrich, UK)	100x	100U/ml 100µg/ml	1ml	Effective against Gram-positive and negative bacteria
N2 (Invitrogen)	100x	1x	1ml	Recommended for growth and expression of neuroblastoma for the survival and expression of post-

				mitotic neurons in primary cultures from both peripheral and central nervous systems.
B27 (Invitrogen)	50x	1x	2ml	Supports the growth of neuronal cells without astrocyte feeder layers and is effective for the growth of neuronal tumour cell lines
EGF 200 µg/ml (R&D Systems®)	10000x	50ng/ml	2.5µL/5ml	Regulation of cell growth, proliferation and differentiation
Noggin 100 µg/ml (R&D Systems®)	1000x	100ng/ml	1µL/ml	Induces expansion of crypt structures
R-spondin1-250µg/ml (R&DSystems®)	1000x	500ng/ml	1µL/ml	A Wnt agonist that induces marked crypt hyperplasia in vivo

Table 2. 6. Antibiotics and other supplements

Solutions	Stock solution	Working concentration
Congo red	1% (v/v)	10µl/ml
Gentamycin (GM)	50mg/ml	50µg/ml

Ampicillin (AM) (Sigma Aldrich, UK)	200mg/ml	200µg/ml
Amphotericin B	250 µg/ml (100X)	2.5µg/ml
Erythromycin (CalBiochem^{R})	50mg/ml	100µg/ml
IPTG (Sigma Aldrich, UK)	1 M	0.1mM
L-arginine methyl ester (L-name) (Sigma Aldrich, UK)	200Mm	5mM
Chloramphenicol (Sigma Aldrich, UK)	100mg/ml	20 µg/ml
Sodium citrate (Signa Aldrich, UK)	1M	5,40mM
$MgCl_2$ (Sigma Aldrich, UK)	1M	20mM
L- (+)-Arabinose (Sigma Aldrich, UK)	1M	200mM
Penicillin / streptomycin (Sigma Aldrich, UK)	10000 unit and 10mg streptomycin/ml	100U/0.1mg
Kanamycin Sulfate (CalBiochem^{R})	100mg/ml	50µg/ml

MTT (Sigma Aldrich, UK)	5mg/ml	100µg/ml

NOTE: all antibiotics were from Sigma Aldrich unless it was indicated.

2. 3. Bacterial strains and plasmids used in this study

All bacterial strains and plasmids and their origin used in the present work are listed in table 2.6. Bacteria were grown routinely on Lysogeny (LB) broth and agar containing 0.1% Congo red for shigella strains; while LB agar or broth for AIEC. All strains were incubated at 37 ˚C overnight to isolate a single colony for the experiments.

Table 2. 7. Abbreviation of bacterial strains, plasmids, and their characteristics and source.

Strains or Plasmids	**Relevant characteristics**	**Size of the plasmid (bp)**	**Growth Temp.**	**References**
LF82, HH427 & HM605 (WT)	Adherent and invasive *E. coli*		Variable	(Zhang *et al.*, 2015)
HHMD	HH427/ *ΔdsbA*		37 ˚C	This study
HMMD	HM605/ *Δ dsbA*		37 ˚C	This study

HMP9	HM605/ *dsbA*-pJYu9		37 ˚C	This study; pJYU9 expresses wildtype *dsbA*
SSWT	*Shigella sonnei* (WT)		37 ˚C	(Xu *et al.*, 2014)
SSMD	*Shigella sonnei/ ΔdsbA*		37 ˚C	(Mirza *et al.*, 2018)
SSPQ	*Shigella sonnei/ ΔproQ*		37 ˚C	This study
SSPQ/pTasy	*Shigella sonnei/ proQ*-pGEM Tasy		37 ˚C	This study, wild type *proQ* coding sequence was cloned into pGEM-T-Easy
ΔMxiD	*Shigella sonnei/ ΔMxiD*		37 ˚C	(Mahmoud *et al.*, 2016)
ΔicsA	*Shigella sonnei/ ΔicsA*		37 ˚C	(Mahmoud *et al.*, 2016)
Top 10	ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK λ– rpsL(StrR) endA1 nupG			(Liu *et al.*, 2015)
DH5α	endA1 hsdR17(rK– mK+) supE44 thi-1 recA1 gyrA (Na1r) relA1 Δ(lacZYA-		37 ˚C	(Maloy & Hughes, 2007)

	argF) U169 (m80lacZΔM15)			
pKD4	A template plasmid for amplification of the Kac cassette	3267	30 ˚C	(Datsenko & Wanner, 2000)
pKD3	A template plasmid for amplification of the chloramphenicol cassette	2804	30 ˚C	(Datsenko & Wanner, 2000)
pCP20	A plasmid expressing the FTR recombinase	9332	30 ˚C	(Datsenko & Wanner, 2000)
pREDTAI	A plasmid expressing the red λ recombinase	6300	30 ˚C	(Yang *et al.*, 2014)
pTRKH3-ermGFP	A plasmid expressing ermGFP	7493	37°C	(Lizier *et al.*, 2010)
PET-GFP	A plasmid expressing GFP	5422	37°C	(Zuris *et al.*, 2015)
PBS-erm GFP	A plasmid expressing ermGFP	4198	37°C	(Lizier *et al.*, 2010)

2. 4. Minimum inhibitory concentration (MIC) of geraniol

An overnight bacterial culture (250 µl) was inoculated in Mueller-Hinton broth 2 (10 ml) and incubated at 37 °C in a shaker until OD600 = 0.3. Following dilution to OD_{600} = 0.1, a further 1:10 dilution was performed in sterile Mueller-Hinton broth2 to give approximately 10^7 CFU/ml accordingly. Sterile Mueller-Hinton broth 2 (100 µl) was then added to each well of a 96-well plate.

A solution of 1M of geraniol in dimethyl sulfoxide (DMSO) was used to prepare a series of dilutions in sterile Mueller-Hinton medium in a 96-well microtiter, beginning in column 3 and continuing to column 12, giving 100 µl of medium per well. Wells 1 and 2 were labeled as negative and positive controls, respectively, to establish bacterial viability in the experimental test.

Hence, 5 µl inoculum of the 1:10 diluted bacterial culture was added to each well, giving approximately $5x10^4$ or 10^5 CFU/well. Following 18 – 20 h incubation at 37°C, the wells were visually examined for turbidity (bacterial growth). A SpectraMax 190 was also used to measure the absorbance at 600nm. The MIC of geraniol was evaluated as the minimum concentration giving no visible signs of growth.

2. 5. Protocol for the bacterial growth curve

The protocol was modified from JoVE (2019) and vlab.amrita.edu (2016). A single isolated colony of each bacterial strain was inoculated into 2 ml of L-broth incubated overnight at 37°C with shaking 200rpm. This relatively long incubation period results in a stationary phase population of approximately 10^9 CFU/ml. On the following day, subcultures were set at OD_{600} = 0.05 for each of the strains and incubated at 37°C with shaking 250rpm. Then, OD600nm was taken every 30min until all cultures reached stationary phase OD600nm + 2.0.

2. 6. Protocol for Biofilm information

The protocol was modified from Nickerson *et al.* (2017). Bacterial cultures from a mid-log phase, approximately OD_{600}nm = 0.3 were diluted further in fresh L-broth containing bile salt (0.2% wt/vol) to 0.05 to give rise approximately $5x10^4$ or 10^5 CFU to each well of the 96-well plate. The plates were incubated at 37°C. After 24 h incubation, the bacterial culture was discarded, and the wells were washed with sterile distilled water two times. 100 µl of 0.5% crystal violet (CV) was added to each well, and the plates were incubated for 15 min at ambient temperature. After CV was discarded the wells were washed with distilled water three times. 150 µl of absolute ethanol was then added in each well and incubated for 10 min at ambient temperature to dissolve the CV-stained biofilms. The dissolved CV-stained biofilm was measured at OD_{540}nm using the SpectraMax Pro. After 48 h incubation, a second set of plates was treated the same way as above to obtain the OD_{540}nm measurements.

To test the activity of geraniol on biofilm formation, a serial dilution of geraniol was supplemented in the cultures and the experimental condition and procedure were the same as above.

2. 7. Methods for Molecular biology

2. 7. 1. Plasmid Extraction (miniprep)

Plasmid DNA was extracted using an Isolate II plasmid mini kit (Bio Line) according to the manufacturer's instructions. Bacteria were harvested from 1 ml overnight culture by centrifugation at 11000 xg for 5 min. The bacterial pellet was resuspended with 250 µl Buffer P1 (containing RNase A). 250µl of lysis buffer P2 was added and the tube was inverted several times and incubated at room temp for 5 min. Then 300µl of neutralization buffer P3 was added with mixing by inverting the tube 4-6times. The sample was centrifuged at 11000 xg for 5min at room temperature. The supernatant was then carefully poured into a spin column. The column was centrifuged at 11000

xg for 1min and the flow-through was discarded. 500 µl of Wash buffer (PW1) was added followed by centrifugation at 11000 xg for 1min and the flow-through discarded. Next, 600µl of wash buffer PW2 (containing absolute ethanol) was added and centrifugated at 11000 xg for 1 min. The flow-through was discarded and the column was centrifuged again for an additional 2min to remove residual wash ethanol. The spin column was transferred to a new microcentrifuge tube and 30-50µl of elution buffer was added to the column and the sample incubated for 2-5min at room temperature; then centrifuged at 11000 xg for 1min to collect the plasmid DNA. The DNA concentration and quality were determined by measuring 260/280 and 260/230 using a Nanodrop device (Thermo Scientific UK).

2. 7. 2. Alteration of pGEM-T-Easy and pBS-ermGFP for chloramphenicol marker (CmR)

Cm^R cassette was PCR-amplified using pKD3 as the template and primers BgLII_Cm Table 2.9 (Cm1: 5'-GGGAGATCTGTGTAGGCTGGAGCTGCTTC- '3; Cm2:5'-GGGAGATCTATGGGAATTAGCCATGGTCC-'3). The amplicon contained the promoter and the entire coding sequence of the cat gene. Primers 9 and 10 introduced BglII restriction sites in both ends of the Cm-amplicon, which were digested with BglII. Inverse PCR was performed on plasmids, pGEM-T-Easy, and pBS-erm, using primers 9 and 10. The inverse PCR products omitted the entire AMP coding sequence and had BglII restriction sites on both ends. The inverse PCR products and the Cm-amplicon was digested with BglII and ligated together. The ligation reaction comprised 500ng DNA insert (Cm PCR product), 50ng vector, 1µl T4 ligase, 1.5µl 10x buffer and nuclease-free H_2O to 15µl. The ligation mix was incubated at 16°C overnight. Electroporation was performed with a mixture of 4µl ligation reaction and 70µl competent *E. coli* DH5α, using GenePulse (BioRad). SOC medium was used to culture bacteria at 37°C for 2 h. Cm resistant colonies were selected by 24-hours incubation on LB-agar containing chloramphenicol (20µg/ml) at 37°C overnight.

2. 7. 3. Genomic DNA extraction from bacteria

Genomic DNA is isolated through the following procedure. Bacteria were harvested from 1 ml overnight culture by centrifugation at 13,000–16,000 × g for 2min. The supernatant is discarded and 600µl nuclei lysis solution was added, with gentle pipetting until bacterial suspension became homogeneous. Cell lysis is obtained after five-minute incubation at 80°C. After cooling to ambient temperature, 3µl RNase solution to the cell lysate, and mixed by inversion of the tube 2-5 times. After incubation for 15-60 minutes at 37°C, the sample is cooled to ambient temperature. The cell lysate was treated with 200 µl solution of protein precipitation and mixed by robust 20-second vortexing at high speed. The sample was incubated on ice for 5 min followed by 3 min centrifugation at 13,000–16,000 × g. The DNA-containing supernatant is moved to a clean 1.5ml microcentrifuge tube with 600µl room temperature isopropanol. The sample was gently inverted a few times until a discernible mass of thread-like DNA strands appeared. Followed 2 min centrifugation at 13,000–16,000 × g, the supernatant was poured off with care. The DNA pellet was washed with 600µl of room temperature 70% ethanol with repetitive gentle inversion of the tube. After 2 min centrifugation at 13,000–16,000 × g ethanol was carefully poured off, and the pellet was left for air-drying for 10-15 minutes. The pellet was dissolved in 100µl DNA rehydration solution with 60 min incubation at 65°C. The DNA concentration and quality were determined using the Nanodrop device as above.

2. 7. 4. Competent cell preparation

500 µl overnight culture has added a flask containing 50 ml LB-broth. After 3h incubation at 37°C, culture reached about OD600nm = 0.5-0.6. Bacteria were harvested by 10 min centrifugation at 4000 xg and 4°C. The supernatant was removed, and the pellet was washed twice with 25 ml ice-cold sterile dH_2O and once

with ice-cold sterile (10%) glycerol. The pellet was resuspended in 400µl, and aliquoted 50-70µl per aliquot for either use immediately or storage at -80°C.

To prepare competent cells for gene knockout using strains containing pKD46 or pKDREG or pCP20, the cultures were incubated at 30°C. To induce the expression of red λ recombinase from pKD46 or pKDREG the cultures were supplemented with 200 µM arabinose 20 µM $MgCl_2$. The preparation steps were the same.

2. 7. 5. Gene knockout. The phage λ red recombination system was employed for the assembly of gene deletions in the adherent invasive *E. coli* background (Datsenko & Wanner, 2000).

2. 7. 6. Primers

The synthesis of primers was undertaken in integrated DNA technologies (IDT) in table 2. 8.

Table. 2. 8. Primers used in the gene knockout.

Primer names	Size of fragment	Annealing	Polymerase	purpose	Sequences

dsbA_	71-72	45 ℃	MyTaq™	Mutation	1,2:F1- 5’ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAG CGTTTAGCGC GTGTAGGCTGGAGCTGCTTC -3’ R1 5’TTATTTTTTCTCGGACAGATATTTCACTGTATCAGCATACT GCTGAACAATGGGAATTAGCCATGGTCC-3’ Phosphorylated-m-F atgaaaaagatttggctggcgctggctggtttagttttagcgtttagcgcatcgg cggcgcagtatgaaGTGTAGGCTGGAGCTGCTTC Phosphorylated-m-R tatttttttctcggacagatatttcacagtatcagcatactgctgaacaaaaacat ccatattgctggtATGGGAATTAGCCATGGTCC
dsbA	16-27	45 ℃	MyTaq™	sequencing	3,4: 5’ATGAACAAAATATTTAAAGTTATCTGG-3’ 5’TTACCACTGAATACCGGCC3’
Yad A like	70	65 ℃	MyTaq™	PCR	5,6: m_F_atgaacaaaatatttaaagttatctggaatccggcaacaggtagttac acGTGTAGGCTGGAGCTGCTTC -‘3. m_R_ttaccactgaataccggcaccgagcgcggcggagtattcaccctggct atATGGGAATTAGCCATGGTCC -‘3

Yad A	18-27	45 ℃	MyTaq™	Cloning	7,8 LF82yadA_F _Atgaacaaaatatttaaagttatctgg LF82yadA_R _ttaccactgaataccggc
pKD46 (without	27-29	60 ℃	MyTaq™	PCR	9,10: pKD46_inv_1 5'GGGAGATCTTTATTGTCTCATGAGCGG3' pKD46_inv_2 5'GGGAGATCTCTCATGACCAAAATCCCTTA'3
BgLII_Cm	29	58℃	MyTaq™	PCR	11,12 Cm1: 5'GGGAGATCTGTGTAGGCTGGAGCTGCTTC -'3 Cm2: 5'- GGGAGATCTATGGGAATTAGCCATGGTCC-'3
proQ	72-73	64	MyTaq™	PCR	13,14m_F- ATGGAAAATCAACCTAAGTTGAATAGCAGTAAAGAAGTAAT CGCGTTTCTGTGTGTAGGCTGGAGCTGCTTCG M_R- TCAGAACACCAGGTGTTCTGCGCGCACAATCAAAGACATAC CCGAATTCAGTATGGGAATTAGCCATGGTCC
proQ	18-21	48	MyTaq™	Sequencin	15,16 F2-CTACGTCCGTTGTAATCAGGA R2-AACATGTTCATGCCTGGC

NOTE: Highlighted bases are complementary to the FRT sequences of the Kan cassette.

The primers 1, 2 and 13, 14 were used in PCR amplification of the Kan cassette. The primers had 20 bases on the 3'-end which was complementary to the FRT sequences that were flanking the Kan-gene, and 50-70 bases at the 5'-end which were complementary to the 5'- or 3' end of the target genes to be deleted. A typical PCR reaction for amplification of the Kan cassette was the following:

Components	Final volumes	Note
*MyTaq Reaction Buffer (5x)	10µl	*The buffer must be vortex before used because of precipitation of buffer component.
Forward primer (100µM) (IDT)	1µl	
Reverse primer (100µM) (IDT)	1µl	
Template DNA (genomic DNA) pKD4- (kanamycin cassette)	2µl	
MyTaq™ DNA Polymerase	1µl	
The total volume up to 50µl		

The PCR cycles were set as following

Temperature	Time	Number of cycles	Outcome	Notes
95˚C	30sec	1	Initial denaturation	* The annealing temperature for *dsbA* is 45˚C while for the other primers is variable
95˚C	40sec	29	Denaturation	
*45˚C	35sec	29	Annealing	
72˚C	30sec	29	Extension	
72˚C	5min	1	Final extension	
4˚C	1hr	1	Hold (completion)	

Dpnl restriction digestion was set to treat the PCR products

Components	Final Concentration
Buffer 5x	5µl
A DNA fragment of plasmid 152.8ng	45µl
Restriction enzyme	1 µl
Incubated at 37 ˚C for 2.303hr or overnight	

To determine the size of the PCR products, agarose gel electrophoresis was performed. The correct DNA bands were then isolated from the gel using the Bioline gel extraction kit according to the manufacture's instruction. Briefly, DNA bands were sliced from the agarose gel and transferred to a microtube. 200µl binding buffer was added to the tube for at 50°C until gel dissolved. The sample was loaded on a spin column and centrifuged at 11000 x g for 60 seconds. The flow-through was discarded and the column was washed with 700 µl wash buffer by centrifugation for 60 sec. The column was dried by a further 2 min centrifugation. 15-30µl elution buffer was added to the column and incubated at room temperature for 2-5 min. The DNA was obtained by 1 min centrifugation at 11000 xg. The DNA concentration and quality were determined by a nanodrop device (Thermo Scientific).

The purified Kan cassette was electroporated into competent cells carrying either pKD46 or pKDREG. The recombinants were selected on L-agar containing 50 µg/ml kanamycin. PCR then was performed to confirm the integration of the Kan cassette in the correct genome location using primers 3 and 4 for *dsbA* and 15 and 16 for *proQ*. Confirmed *dsbA*-kan and *proQ*-kan mutants were grown at 40°C to eliminate pKD46 or pKDREG. The plasmid-cured kan-resistant mutants were transformed with pCP20, which expressed the FRT recombinase that promotes the FRT recombination to remove the Kan-cassette. After confirmation of the removal of the Kan-cassette by reinoculating on L-agar containing 50 µg/ml kanamycin, the strains were grown at 42°C to cure pCP20. The deletion of the genes was confirmed by colony-PCR using primers 3 and 4 for *dsbA* and 15 and 16 for *proQ*.

2. 8. Culture mouse raw macrophage cell-line and assays

2. 8.1. Raw 264.7 macrophages cell-line and maintenance

The murine macrophage-like cell line raw 264.7 (ECACC 91062702) was obtained from the European Collection of Authenticated Cell Cultures (Public Health Laboratory Service). The cell-line was routinely cultured in supplemented DMED (Table 2.3) at 37°C, with 95% humidity and 5% (v/v) CO_2. Cells were left to grow until they reached 80% confluency and scrapped off for passage or seeded for the experiments described below.

2. 8. 2. Gentamycin protection assay

Cells were seeded into 24-well plates, 10^5 cells/well, 24 h before the experiment. For imaging purposes, cells were seeded into 24-well plates which contained a glass coverslip in each well. Next day fresh DMEM was used to replace the spent one for 30-60 min. Bacteria from mid-log phase (OD_{600}nm = 0.3) was added to the cell-monolayer with the multiplicity of infection (MOI) = 10 (10 bacteria per cell) or MOI = 100. Bacteria were allowed to invade the cells for 50-60 min at 37˚C under 5% CO_2. The medium was then removed, and cells were washed with 1X PBS three times. One ml gentamicin containing DMEM (50 μg/ml) was added to each well, followed by appropriate incubation intervals 37˚C under 5% CO_2. The medium was removed, and cells were washed with 1X PBS three times. For enumeration of intracellular colony-forming units, cells were lysed with 1ml of 0.1% (v/v) Triton and lysate (with appropriate dilutions) were plated out on L-agar. For microscopical examination, the cells were fixed on the coverslips with paraformaldehyde (PFA; 3.7%) for 15 min. After removal of PFA, coverslips were washed with 1X PBS and treated with MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (100μg/ml) and incubated 30 – 60 min. The MTT solution was taken off and replaced with PBS. Cells were examined and images were taken under the Evo's microscope (Life Technology Evos FL Auto).

The gentamicin protection assay was used for evaluating the effect of geraniol as the antimicrobials. Raw cells were infected the same way as above in 24-well plates. During the incubation with gentamicin containing DMEM, the medium was additionally supplemented with various concentrations of geraniol. If the geraniol had activity against the intracellular bacteria, the effect would directly indicate by the reduction of CFU.

2. 8. 3. Raw cells for evaluation of viability

Live cells may reduce MTT in a mitochondrial-dependent manner to form insoluble formazan and became whereas dead cells remained colourless. Thus, the formation of formazan offers quantitative measurement of live/dead cells. In doing so, 200 µl of raw macrophages (6.72 x 10^5/ml) were seeded each well of the 96-wells plates and allowed to grow overnight. The next day, the spent medium was removed and DMEM supplemented with different concentrations of geraniol was applied to the cells. Followed by overnight incubation at 37°C and 5% CO_2, cells were washed with 1X PBS and 100 µl of MTT solution was added to each well, which was covered and incubated until purple precipitation occurred. After removing the MTT, cells were washed with 1X PBS and the formazan was extracted with DMSO (100 µl/well). A Spectramax 190 microplate reader was used to measure absorption at 595nm.

2. 8. 4. Production of Nitric oxide (NO) from Raw cells

A method modified of Min *et al.* (2009) was used. Briefly, Raw cells were seeded in 24-well plates with 1 × 10^6 cells/well and incubated for 24 h at 37°C under 5% CO_2. Cells were infected and treated with various agents (geraniol, or L-NAME, or LPS) as per 2.8.2, for 24h at 37°C under 5% CO_2. The amount of NO in the culture medium was measured by the Griess reagent (conversion of nitrate to nitrite by nitrate reductase which reacts with sulfanilic acid and N -(1-naphthyl) ethylenediamine to

form an azo-dye that can be measured at 540 nm. A standard curve was prepared with a known concentration of sodium nitrite ($NaNO_2$) plotted against absorption at 540nm.

To test the activity of the standard and other reagents the present work follows:

Firstly, prepare twelve Eppendorf tubes and label with a number, the last-mentioned with Blank (just media). Then add 990 µl of media that used for a macrophage to the first tube and 500 µl for the others for serial dilution. Next, 10 µl of sodium nitrite was added to the first tube and mixed properly and then each time a 500 µl were taken to the next tubes until throughout a 500 µl from the one before the blank to generated (100 to 0.09765625 µM) concentrations. Later, a 96 well/ plate was prepared and labelled with the same number. To 50 µl of nitrate was added 50 µl of Griess reagent and incubated for 5 minutes and then the absorbance determined at 540nm (Jie Sun **2003;** Sun *et al.*, 2003).

NO-Standard

	1	2	3	4	5	6	7	8	9	10	11	12
A	84 µM Geraniol	25mM L-name	Positive control (Infect. Macrophage)	Neg. control (macrophage)	1 µg LPS	25mM-L-name + 1µg LPS						
B	84 µM Geraniol	25mM L-name	Positive control (Infect. Macrophage)	Neg. control (macrophage)	1µg LPS	25Mm—L-name + 1µg LPS						
C	84 µM Geraniol	25mM L-name	Positive control (Infect. Macrophage)	Neg. control (macrophage)	1µg LPS	25mM - L-name + 1µg LPS						
D												
E												
F												
G	100µM	50µM	25 µM	12.5 µM	6.25uM	3.125µM	1.6525µM	0.78125µM	0.390625µM	0.1953125 µM	0.1953125 µM	Blank (media)
H	100µM	50µM	25 µM	12.5 µM	6.25uM	3.125µM	1.6525µM	0.78125µM	0.390625µM	0.1953125 µM	0.1953125 µM	Blank (media)

Figure 2. 1. A diagram indicated the distribution of the components of NO assay as follow:

1. Infected macrophage treated with 84 µM of geraniol.

2. Infected macrophage treated with 25 mM of L-name (inhibitor for NO production).
3. Infected macrophages non- treated (Positive Control).
4. Non- infected macrophage (just macrophage) (Negative control).
5. Macrophages treated with 1µg LPS of *E. coli.*
6. Macrophages stimulated with 1µg LPS of *E. coli* & treated with 25mM of L-name.
7. Macrophage stimulated with 1µg LPS of *E. coli* and treated with 84 µM of geraniol.
8. G & H. A serial dilution in the same media used in the above sample and bank for the No- standard prepared according to (Standard of Griess assay).

2. 9. *G. mellonella* larvae model

The moth larvae were purchased from LiveFood UK. Larvae were approximately 250-300 mg each. Bacteria collected from mid-log phase were diluted with 1xPBS to 10^5, 10^6, $10^7$and 10^8 CFU/ml which is almost equivalent to OD= inapplicable, 0.01, 0.02, 0.13 respectively based on the free online Labtools; bacterial cell number (OD=600nm) (https://www.labtools.us/bacterial-cell-number-od600/), and 10µl of the bacterial suspension was injected to the rear proleg to achieve the doses 10^5, 10^6 and 10^7 CFU/larvae. The larvae were incubated at 37°C in the dark after injection. Larvae observed twice daily for pigmentation and death over five days. Larval death was scored if the larvae did not respond to touch. Every experiment was repeated separately a minimum of three times. The Kaplan-Meier estimator was employed for the examination of the survival data.

2. 10. Intestinal organoid model

2. 10. 1. Mouse dissection and statement of ethics

All animal work was approved by the Glasgow and Strathclyde University Committee on Animal Resources. Mice were euthanised by carbon dioxide asphyxiation or cervical dislocation as recommended by the 2000 Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia in accordance with Home office Conditions (AVMA, 2001; Leary, 2013). The abdomen of the animal was sterilised with 70% (v/v) ethanol. Then, the skin and abdominal wall were dissected, and 15 cm of the proximal small intestine removed where the cardiac sphincter and end of the small intestine were detected and separated from the mesentery and other abdominal organs (O'Rourke *et al.*, 2016). Finally, the mouse small intestine was removed immediately and kept in PBS (without Ca^{2+} and Mg^{2+}).

2. 10. 2. Isolation of small intestinal crypts

The small intestine was cut longitudinally into 5 mm pieces, placed in a petri dish; washed three times with PBS to remove any faecal material and luminal contents. The pieces were transferred into a 50ml Falcon tube containing 15-20 ml PBS with 2mM EDTA. This was kept on the ice for 25-30 min. Small intestinal crypts were separated from the rest of the tissue using a 15-20 ml dissociation (shaking) buffer with gentle shaking 3-5 min at 2-3 cycles per second (to dissociate the epithelium from the basement membrane). This fraction was passed through a 70 µm filter to remove the villi. The sample was centrifuged at 150 xg and 4°C for 10 min and the supernatant was discarded. The pellet was resuspended in 2-5 ml PBS and shake gently. The crypts were counted using a haemocytometer. Appropriate numbers of crypts were mixed with the growth factor in 0.5ml Matrigel and immediately placed in the centre of a well of a pre-warmed 24-well plate. The plate was incubated at 37°C for 10-20 min to allow polymerization of the Matrigel. Thereafter, 0.5 ml of complete

organoid growth medium (Table 2.4) was added and the plate incubated at 37°C under 5% CO_2 overnight.

2. 10. 3. Passage (splitting) of the organoids

Complete organoid growth medium was replaced every 2-3 days, and mature organoids were passaged every 7 days. To do this, the medium was removed from each well and the Matrigel dome broke up using a P1000 pipette. When it was sufficiently broken, it was pipetted several times against the bottom of the plate to break up the crypts. The same pipette tip was then used to flush the well twice with 0.5 ml PBS. The crypt-media solution was then centrifuged at 150xg for 10 min at 4°C, and the pellet resuspended with Matrigel contains growth factors and re-plated as described above.

2. 10. 4. Evaluation of the viability of organoids by geraniol by MTT reduction

Organoids were cultured for 3 – 4 days in a 96-well plate as above. They were then incubated with complete organoid growth medium supplemented with various concentrations of geraniol (5.5 µM – 2688 µM). Following 24 h of incubation under 5% CO_2 at 37 °C, the media were removed and the MTT solution was added to the culture, giving a final concentration of 5mg/ml. After incubation for 2 – 3 h under 5% CO_2 at 37 °C, the medium was removed and 20 µl of the SDS solution (2%) was added, and a further 1 h incubation at 37°C to solubilise the Matrigel. The insoluble formazan was then dissolved by the addition of DMSO (100 µl) for 1 h at 37°C. A SpectraMax 190 microplate absorbance reader was then employed to measure the OD at 562nm. Following the protocol of (Grabinger *et al.*, 2014), the 0% viability control was 15 µl of unpopulated Matrigel, while 100% viability was defined using untreated organoids.

2. 10. 5. Direct infection of organoid cells

A modified protocol proposed from Zhang *et al.* (2014) was used to induce infection in the organoid cells six days after passage. Colonisation of the cells with the specific *Shigella* and AIEC (wild type, *ΔdsbA* mutant, transformed pJYu9 plasmid) strains containing the PBS-erm GFP plasmid was conducted over a period of half an hour at MOI 10^7. Subsequently, PBS was used to wash the cells a single time. The cells were then subjected to 60-minute incubation in mini gut media that contained 50 mg/mL gentamicin, which prevented extracellular bacteria from growing (Sun *et al.*, 2004). Following, the addition of 1ml of 0.1% triton to every well, 15-minute incubation at 37°C was conducted. The next step was plating 100µl lysate on LB and overnight incubation at 37°C to determine how many bacteria there were on each plate.

The addition of 100µg/ml MTT to the culture medium was done in the final half-hour of one hour after infection. This was done in order to be able to use Evo's microscope to observe the bacteria and any alterations in organoid form and structure.

2. 10. 6. Co-culture organoids with infected Raw cells

Raw cell monolayers were infected for 2h as per 2.10.2 and washed thoroughly with 1XPBS. Organoids were split as per 2.10.3. and placed on the Raw cell monolayers and incubated for a period of 20 min at 37˚C under 5% CO_2. Then, a 0.5 ml complete organoid medium supplemented with gentamicin (50 µg/ml) was added and incubated overnight at 37˚C. Live and dead organoids were counted using an Olympus MI microscope (Japan). Images were taken using an epi-fluorescent inverted microscope (Nikon Eclipse TE300) in order to ascertain the disparities of the normal organoid and macrophage, and macrophage infected with organoid.

2. 10. 7. Co-culture of Raw cells and organoids and treatment with cytokines and LPS

Raw cells were added to the 24-well plate (1x 10^5/well) containing 3-4 days of age organoids after they are split. The cells were incubated overnight at 37°C in order to settle. Subsequently, the cells and organoids were exposed to 50 ng of IFN-alpha, 100ng/ml of TNF- gamma, and 1 µg of LPS of *E. coli* and incubated overnight at 37°C with (5%) carbon dioxide. To distinguish live and dead organoids after exposure cytokines or LPS to the Raw cell/organoids they were stained by addition of (0.4%) trypan blue solution (40 µl) to the media (400 µl) and incubated under 5% CO_2 for 10 – 20 min at 37 °C. Cell viability relative to the untreated controls was then determined by counting the living and dead organoids under the Olympus MI microscope (Japan). Images of the treated and untreated organoids were captured using an epifluorescent microscope (Nikon Eclipse TE300).

2. 11. Statistical analysis

All results were reported as mean ± SD. The student ANOVA test (Tukey test), Bonferroni, t. test and Kaplan-Meier survival curves were applied to determine statistical significance in various experiments where appropriate. All graphs and figures were generated with GraphPad Prism version 5.0, except the growth curve and biofilm treated with geraniol using Excel.

3. Chapter Three. Construction and characterisation of mutant *S. sonnei* and AIEC strains

3. 1. Introduction

3. 1. 1. Molecular Koch's postulates – usefulness and limitations in studying microbial virulence

Koch's postulates were proposed in the year 1882 (Breitschwerdt *et al.*, 2013). The postulates offered a guide for the identification of specific causes of infectious disease and, in addition, were aimed at persuading sceptics that microorganisms could cause diseases. In brief, the disease and its characteristic lesions must be regularly associated with the organism, the organism must be isolated and cultivated from the diseased host, and the infection must be replicated when a pure culture of the organism is introduced into a stable, susceptible host (Tabrah, 2011). Koch's postulates became the basis for defining the pathogenic role of a microorganism in a disease process and received strong support from the scientific community. However, Koch's postulates have limited applications. For example, a single pathogen, such as *Bacillus anthracis*, can be responsible for several disease conditions. Conversely, multiple different microorganisms can cause a single disease condition, such as tuberculosis. A major challenge is that there are many uncultivable microorganisms, and some pathogens, such as human immunodeficiency virus (HIV), cause disease only in humans, which cannot be tested in animal models (May, 2011; Nina Parker, 2016).

As modern medicine entered the molecular era, Molecular Koch's postulates were proposed by Falkow (1988) and were recently reviewed by Falkow (2004). The postulates included that (1) the phenotype or pathogenic properties of an organism under investigation should be correlated with a particular individual genus, (2) the direct inactivation of the gene(s) should lead to testable attenuation of the pathogenicity, (3) the reversal of the mutated gene must result in the restoration of

pathogenicity. Currently it is not possible to apply Koch's postulates to a gene in question for many pathogenic microorganisms since important genes for pathogenic microorganism metabolisms obviously do not play a role in pathogenicity or virulence (Falkow, 1988). Testing a candidate virulence gene requires an appropriate animal model for the disease being examined and the ability to genetically manipulate the disease-causing microorganism. Suitable animal models for many major human diseases are lacking. In contrast, it is not possible to genetically manipulate most pathogens (LibreTexts, 2019). In accordance with an improved understanding of the nature of microbial pathogenesis, especially as it occurs in association with fastidious or unculturable species, polymicrobial infections, and chronic or recurrent infections, the number of postulates has continued to increase (Breitschwerdt *et al.*, 2013).

In 1996, Fredericks and Relman subsequently proposed Koch's molecular postulates established primarily on the sequence-based classification of microbial pathogens (Fredricks & Relman, 1996). In brief, such methods based on nucleic acids allow the identification of microbes that are associated with a disease but that cannot be cultivated. Nucleic acid-based detection methods are also very flexible and can often detect very low levels of viruses present in stable, disease-free people (Breitschwerdt *et al.*, 2013; Fredricks & Relman, 1996). Such changes are still problematic because they do not account for proven associations of diseases such as papillomavirus and cervical cancer, nor do they take prion diseases (which do not have their own nucleic acid sequences) into account.

To apply molecular Koch’s postulates, this study began with the deletion of genes thought to be important for *Shigella* and AIEC pathogenesis, using the red lambda (λ) recombination system. Mutant and wild type strains and complement strains were characterised by their growth rate and the development of biofilms to assess general fitness and virulence.

3. 1. 2. The red λ system – mechanisms and applications

According to Posfai *et al.* (1999) and Russell *et al.* (1989), bacterial chromosomal genes can be inactivated by general allele replacement methods. Historically, in the *Escherichia coli* (*E. coli*) chromosome, two methods of gene replacement were developed. The first was the 'in-out' process, based on plasmid incorporation into the bacterial chromosome and the subsequent cointegrate resolution. Another process, known as 'linear fragmentation' or 'recombineering', focused on homologous recombination at the ends of a linear DNA molecule, controlled by short homology arms (Madyagol *et al.*, 2011).

Most bacteria are not readily transformable with linear DNA. The existence of intracellular exonucleases, which degrade linear DNA (Lorenz & Wackernagel, 1994), is a reason for the non-transferability of *E. coli*. Electroporation has been shown to enable the internalisation of double-stranded linear DNA in *E. coli* (Feher *et al.*, 2007). Feher *et al.* (2007) further demonstrated that the simple use of PCR-generated fragments was precluded because of the rapid degradation of the DNA by the exonuclease activity of the RecBCD complex. Therefore, a red λ system was created to perform gene knockout in bacteria (as shown below).

For recombineering to occur, the incorporation of phage recombination genes (*Gam*, *Bet*, and *Exo*) is used. The red λ system expresses Exo, Beta, and Gam proteins that work in conjunction to facilitate homologous recombination which integrates double-stranded DNA into the chromosome (Datsenko & Wanner, 2000; Muniyappa & Radding, 1986; Yu *et al.*, 2000). The system has been exploited for genome modifications in *E. coli, Salmonella enterica,* and other Gram-negative bacteria (Court *et al.*, 2002; Murphy, 1998; Yu *et al.*, 2000). Exo has a 5'–3' double-stranded DNA (dsDNA) -dependent exonuclease activity for generating 3' single-stranded DNA (ssDNA) overhangs (Matsuura *et al.*, 2001), which then serve as a substrate for the ssDNA-binding protein Beta to anneal complementary DNA strands for

recombination (Copeland *et al.*, 2001; Karakousis *et al.*, 1998; Muniyappa & Radding, 1986). Gam, an inhibitor of host exonuclease activity due to RecBCD (Poteete *et al.*, 1991), helps to improve the efficiency of λ Red-mediated recombination with linear dsDNA. The basic strategy is to substitute a coding sequence in the chromosomes (e.g. gene B in Figure 3.1A) with a selectable antibiotic resistance gene produced by PCR which is flanked by short flip base recognition (FLP) followed by up- and down-stream of the substituted coding sequence. The resistance gene can be removed using the helper plasmid that expresses the FLP recombinase, which works on the FRT sites flanking the resistance gene. Red and FLP help plasmids can be cured by growth at 42°C simply because they are temperature sensitive for replication (Datsenko & Wanner, 2000). It has been demonstrated by the Subramanian group that binding of the 5'-phosphorylated end of the dsDNA substrate by residues proximal to the catalytic site is a crucial step in the processive resection of linear dsDNA molecules by λ Exo (Subramanian *et al.*, 2003). Therefore, phosphorylation of the PCR products may increase the recombination rate.

3. 1. 3. Bacterial growth as a measurement of fitness

Microbiological fitness is the potential of microbes to survive in a competitive environment. Fitness is often calculated by comparing the rate of growth in a given mutant strain with its isogenic non-mutant relative. If the mutant is less healthy, in specific circumstances it will grow more slowly (Elio, 2019). A typical example is a mutant that, in the presence of an antibiotic concentration, has acquired the ability to grow. Clearly, in the presence of the drug, such a mutant will evolve, but its parent will not. Nevertheless, the parent will outgrow the mutant in the absence of the drug. This is due to a 'fitness cost' that the mutant needs for producing molecules such as enzymes for drug resistance. It is possible that such enzymes would be beneficial if the organism encountered the drug again (Elio, 2019).

One widely used method of quantifying microbial fitness is to measure the maximum growth rate (Walkiewicz *et al.*, 2012), typically by calculating the optical density of the culture over time. Such measurements have the advantage of being simple and quick; in quick succession, a spectrophotometer can measure several samples in a multi-well plate, and systems can be designed to calculate the entire growth cycle of a culture. The maximum growth rate, however, is usually only one aspect of fitness even in the simplest systems (Vasi *et al.*, 1994), and thus, offers at best, only a fitness proxy.

3. 1. 4. Biofilm formation as a measurement of fitness

Other *in vitro* models available for use in the measurement of fitness include quantification of biofilm production, water survival, and drying (Pope *et al.*, 2010). The selected models rely on the species, its natural lifestyle, and its growth pattern. Biofilms, for instance, play a role in many infectious diseases and, for example, the bacteria *Pseudomonas aeruginosa* is known to form biofilms within the human body, such as in the lungs in the case of cystic fibrosis (Pope *et al.*, 2008; Singh *et al.*, 2000). This is because a shift in the ability of the microorganism to produce a biofilm is likely to have a significant impact on its ability to survive or cause infection in the environment. Environmental survival, such as drying resistance or water survival, is likely to be critically important for nosocomial pathogens that can be transmitted through contaminated surfaces under drying pressure (Pope *et al.*, 2008; Sanchez *et al.*, 2002). Fitness deficits can vary depending on the mutation of resistance, the species, and the method used to measure costs. Sanchez *et al.* (2002) measured the fitness in overproduction of multidrug efflux pumps, encoded in *nalB* and *nfxB*, in *P. aeruginosa* using different models such as survival in water, biofilm production, nematode killing, and quantification of proteases.

Competitive analysis between isogenic antibiotic-susceptible and antibiotic-resistant bacteria in culture or in animal models can also determine relative fitness. These

models can be adapted for use in many species of bacteria; biofilm formation and environmental survival models have been used for *Burkholderia cepacia* to determine the fitness value of fluoroquinolone resistance acquisition (Pope *et al.*, 2008).

3. 1. 5. Virulent genes under investigation

In the present study, a chromosomal gene *ProQ of S. sonnei* is homologous to *Salmonella* where it revealed to bacterial motility dysregulation due to *ProQ* is important for interactions with the major flagellin encoding *fliC* mRNA.

Similarly, researchers have also found that the deletion of the *proQ* gene in *Salmonella* leads to a down-regulation of the transcripts of the chemotaxis gene *cheW* and the *ropE* operon (Westermann *et al.*, 2019).

Additionally, the importance of *proQ* to virulence genes accompanied by altered mitogen-activated protein kinase (MAPK) signalling in a host. Where *ProQ* is critical to binding directly to individual SPI-1 transcripts for structural components of the SPI-1 needle or secreted effectors (e.g. *prg* encoding the T3SS-1 *and sicP-sptP* operons, where *sicP-sptP* is transcriptional unit encoding the SPI-1 effector SptP and its chaperone SicP) (Westermann *et al.*, 2019). The deletion of *proQ* causes fewer MAPK signals compared to *Salmonella* wild type. The MAPK signalling cascade is essential to activate the nuclear factor and interferon responses. It is one of the primary targets of bacterial pathogens because they manipulate host immunity to their advantage (Arthur & Ley, 2013). Specific effectors of the SPI-2 regulon, for example, whose expression is reduced in *Salmonella* bacteria, are known MAPK signalling activators in the host, while the SPI-1 effector SptP, whose level increases in the absence of *proQ*, inhibits the MAPK cascade by extracellular signal- kinase (Lin *et al.*, 2003; Mazurkiewicz *et al.*, 2008; Odendall *et al.*, 2012).

Another chromosomal gene is *dsbA* of adherent invasive *E. coli* (AIEC), which has been found to play a major role in the pathogenesis of CD-associated *E. coli.* Removal of *dsbA* in AIEC strain LF82 resulted in pleiotropic effects on the adhesion and eventual invasion of intestinal epithelial cells. Specific virulence factors (such as nutrient-poor growth conditions and/or too acidic growth conditions) post-translationally modified by *dsbA* and/or a specific regulator have recently been found to play a central role in the orchestration of intra-macrophagic survival/replication of AIEC bacteria, a pathogenic characteristic necessary to release large amounts of alpha tumour necrosis (Bringer *et al.*, 2007).

mxiD and *icsA* are genes located in the ≈200-kb virulence plasmid of *S. sonnei* (Mahmoud *et al.*, 2016; Mirza *et al.*, 2018). MxiD is an essential part of the type three secretion system (TTSS) (Allaoui *et al.*, 1993), while IcsA performs dual roles by fostering host cell adhesion in addition to actin-based motility (Brotcke Zumsteg *et al.*, 2014). In the present study, these two genes were investigated under the hypothesis that TTSS and adhesin are important for biofilm formation.

3. 1. 6. The intervention of biofilm formation with geraniol

The use of geraniol as an antimicrobial agent was investigated by De Fazio *et al.* (2016), who reported that oral administration to colitic mice effectively prevented dysbiosis and decreased the systemic inflammatory response.

Most antimicrobial activity of terpenoids is linked to their functional groups, and the hydroxyl group of phenolic terpenoids and the presence of delocalised electrons are important elements for their antimicrobial action. For example, carvacrol is more effective than other EOs such as p-cymene (Ben Arfa *et al.*, 2006; Dorman & Deans, 2000; Ultee *et al.*, 2002). The exchange in carvacrol between the hydroxyl group and a methyl ether can affect hydrophobicity and antimicrobial activity. The position of the hydroxyl group within the phenolic molecule does not affect the antimicrobial action trend (Nazzaro *et al.*, 2013b). In assays, geraniol showed effective activity in

modulating the drug resistance of various Gram-negative bacterial species such as *Enterobacter aerogenes, E. coli,* and *P. aeruginosa* by targeting efflux pumps. It could also restore drug susceptibility in strains with over-expressed efflux pumps (Chouhan *et al.*, 2017).

Geraniol was found to inhibit intracellular *S. sonnei* growth and provided protection to *Galleria* moth larvae from fatal *S. sonnei* infection (Mirza *et al.*, 2018). Therefore, in the present study, the ability of geraniol to reduce biofilm formation was tested, which would potentially impose a different impact on bacterial fitness and virulence.

3. 2. Aims of the experiments

The aims of the experiments were to:

1. Construct and genetically verify mutants bearing individual in-frame deletions in each of the genes of the virulent candidate.

2. Study the impact of gene deletion on bacterial growth.

3. Study the impact of gene deletion on biofilm formation and the effect of geraniol on biofilm formation.

3. 3. Results

3. 3. 1. Construction of the deletion mutants

The plasmids pKD46 and pKDREG were transformed into strains of *S. sonnei* and AIEC, respectively, for the expression of the red λ recombinase (Figure 3. 1 A). pKDREG (chloramphenicol resistance) was used in AIEC, which were resistant to ampicillin. Plasmid pKD4 was used as the template for PCR amplification of Kan cassette. Primers (13, 14 and 1, 2; Table 2. 9) were used for *ProQ* and *dsbA* of *Shigella* and AIEC, respectively.

Construction of an *S. sonnei ΔproQ* mutant was achieved using the method developed by (Datsenko & Wanner, 2000) as described in chapter two (utilising unphosphorylated primers). Using the same procedure, the construction of the *ΔdsbA* mutants in the AIEC strains proved to be difficult. Therefore, phosphorylated primers were made in ITD, which helped to achieve the deletion in the AIEC strains. Verification of the insertions in the *proQ* and *dsbA* genes was achieved using PCR on colonies that were grown on the LB containing 50 µg kanamycin with primers 3, 4 and 15, 16 for *dsbA* and *proQ,* respectively (Table 2. 9). The confirmed Kan resistant colonies were then incubated at 42°C to cure the plasmids pKD46 and pKDREG from *S. sonnei* and AIEC strains, respectively. Kanamycin-sensitive colonies were transformed with the plasmid pCP20 which expressed the FRT recombinase for the second cross-over to loop out of the Kan cassette. Colonies were grown at 42°C to cure pCP20 and then check for loss of Kan cassette. The resultant deletion mutants all had a scar of <200 bp (Figure 3. 1 B).

For complementation of the *S. sonnei ΔdsbA* strain, plasmid pJYU9 was used (Thaer A Hansan, Ph.D. thesis, 2019). The AIEC strains were all ampicillin-resistant, therefore, for complementation of the AIEC *ΔdsbA* mutants, a pJYU9-Cm plasmid was newly engineered, which expressed wild type *dsbA* and conferred chloramphenicol resistance. For complementation of the *S. sonnei ΔproQ* mutant, the wild type *proQ*

gene was cloned into pGEM-T-Easy, and the resultant clone was transformed to the *ΔproQ* strain.

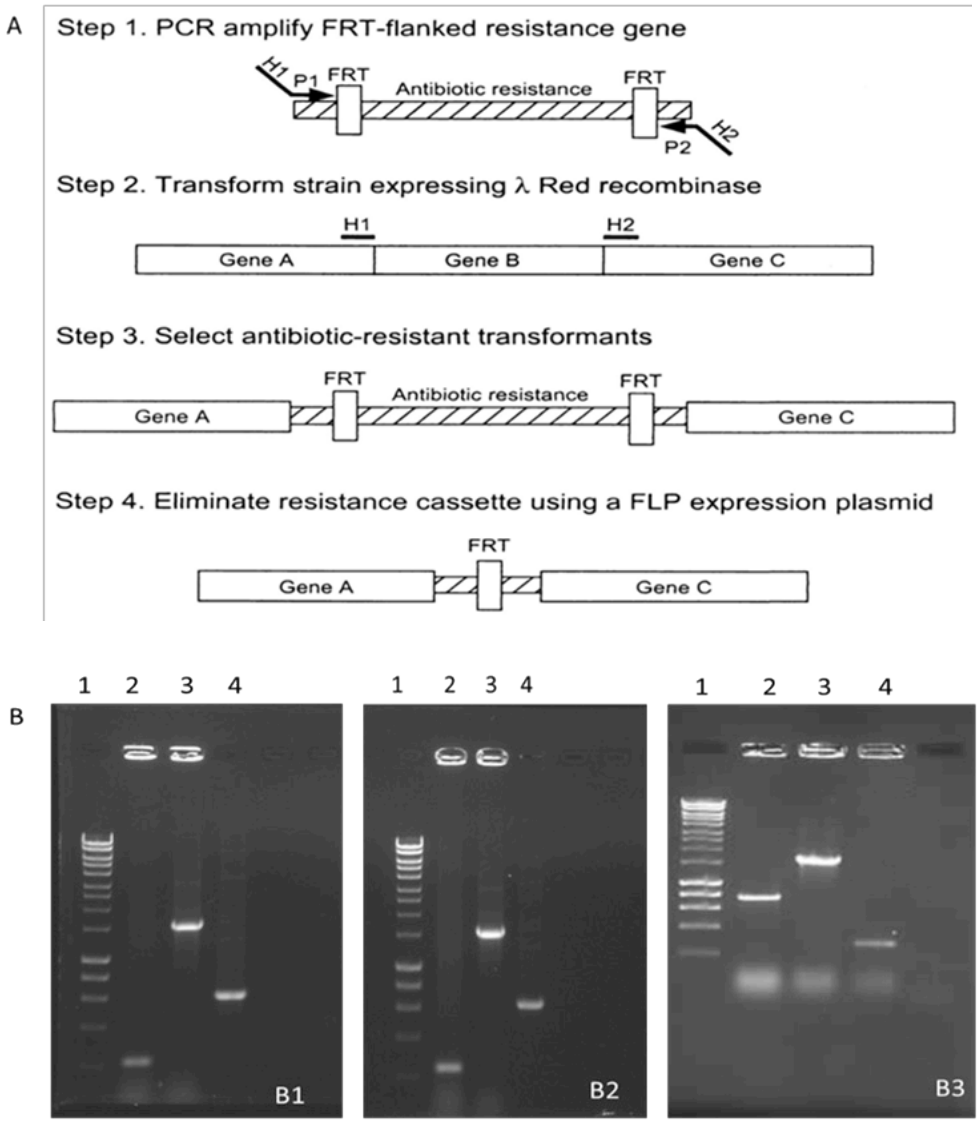


Figure 3. 1. Construction and verification of deletion mutant strains. A. Schematic illustration of the procedure for the construction of gene deletion using the red λ system as originally described (Datsenko & Wanner, 2000). AI: PCR amplification of kanamycin cassette; AII: replacement of the wildtype gene with the Kan-cassette; AIII: removal of the Kan-cassette leaving a 200 bp scar of the gene. Draws are not to scale. **B.** Verification of gene deletions by gel electrophoresis of PCR products. PCR was performed as described in Materials and Methods, and 1% agarose gel was used to separate the PCR products. B1 and B2 show

deletion scars, kan-cassette insertions and wild type genes of *dsbA* (627bp) of AIEC- HM606 and HH427 (lanes 1, 2, and 3). B3 shows the wild type, kan-cassette, and deletion scar of the *proQ* gene (699bp) of *Shigella* (lanes 2, 3, and 4) respectively.

3. 3. 2. Bacterial growth

Bacterial growth was measured in triplicate, three times, and the average of the reading was used to plot a graph. The growth of the *ΔicsA, ΔmxiD, proQ*, and *ΔdsbA* mutant strains was slightly slower (Figure 3.2A&B) but not statistically significant (Table 3. 1) compared to the wild type strains as well as the complement strains. Hence, it was concluded that there was no significant effect of these gene deletions on the general fitness of the *S. sonnei* and AIEC strains.

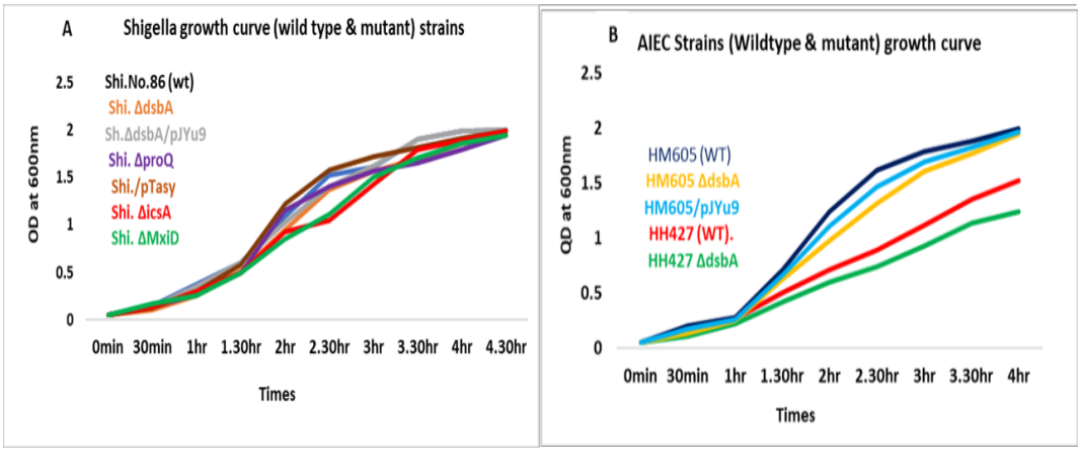


Figure 3. 2. Bacterial growth curves. (A) *Shigella* and (B) AIEC strains. Bacterial growth was measured with a spectrometer OD600nm every 30 min. Bacteria were grown at 37°C in LB broth and incubated for 24 h. The tests were done in triplicate for each strain. The mean value of readings was used to plotted in line graph using Excel.

Table 3.1. Comparison of growth curves

Test pair	Mean	95% CI	*p*-Value
Shi. No 86 (wt) vs Shi. No 86 *ΔdsbA*	0.0810	-0.998 to 1.16	0.238
Shi. No 86 (wt) vs Shi. No *ΔdsbA*/pJYu9	0.01350	-1.08 to 1.08	0.0903
Shi. No 86 (wt) vs Shi. No 86 *ΔproQ*	0.0625	-0.981 to 1.11	0.184
Shi. No 86 (wt) vs Shi. No 86 *ΔproQ*/pProQ	-0.021	-1.06 to 1.02	0.0618
Shi. No 86 *ΔdsbA* vs Shi. No 86 *ΔdsbA*/pJYu9	-0.0775	-1.16 to 1.00	
Shi. No 86 *ΔproQ* vs Shi. No 86 *ΔproQ*/pProQ	-0.083	-1.13 to 0.960	0.246
HM605 (WT) vs HM605 *ΔdsbA*	0.119	-0.825 to 1.06	0.374
HM605(WT) vs HM605 *ΔdsbA* /pJYu9	0.045	-0.899 to 0.989	0.142
HM605 *ΔdsbA* vs HM605 *ΔdsbA* /pJYu9	-0.0739	-1.02 to 0.870	0.233

Table 3.1. A comparison for the growth curve in figure 3. 2 AB was made for all strains with Bonferroni's Multiple Comparison Test of GraphPad Prism software 5, where it was found non-significant.

3. 3. 3. Biofilm formation and MIC

Wild type, *HH427 ΔdsbA*, and *HM605 ΔdsbA* mutants were inoculated in LB medium containing bile salts (0.2% wt/vol) and incubated at 37°C overnight. The formation of the biofilm was measured as described in chapter two (materials and methods). The test was conducted three times and was compared with the wild type and media as negative controls. The *dsbA* mutants showed decreased ability to form a biofilm with the progression of time (Figure 3. 3 A & B), which was statistically significant at 24 hrs (p = 0.0001) and 48 hrs (p = 0.0004 and 0.0001), respectively, compared with the wild type and complement strains (*ΔdsbA*/pJYu9-Cm) (Figure 3. 3 A & B). The experiments were repeated without bile salt in the medium. There was no significant difference in biofilm formation with and without bile salt (data not shown).

Regarding the *S. sonnei* strains, there was also a significant decrease of mutant bacteria compared to the wild type and complement strains (*ΔdsbA*/pJYu9 and *ΔproQ*/pTasy) at 24 hrs (p = 0.0039 and p = 0.0001, respectively) (Figure 3. 3 C). There was decreased biofilm formation after 48 hrs (p = 0.0001 and p = 0.0001, respectively) (Figure 3.3D). The *icsA* and *mixD* mutant strains also showed a significant decrease in biofilm formation compared to the wild type strains after 24 and 48 hrs (p = 0.0001 and p = 0.0004, respectively) (Figure 3.3 E & F).

To establish the effect of geraniol on biofilm formation, a serial dilution of geraniol from 4.2 mM to 0.0328 mM was supplemented to the medium used for culturing wild type *S. sonnei* and AIEC (HH427 & HM605). The results showed that geraniol was capable of inhibiting biofilm formation in a dose-dependent manner, and at 4.2 mM geraniol reduced biofilm formation for all three strains to the level of their respective mutants (please refer to Figure 3. 3 and 3. 4 A, B & C).

The results shown in Figure 3. 4 ABC can be explained by the suggestion that geraniol inhibited biofilm formation (4.2mM) which was 1.5625-fold higher than the concentration that significantly inhibited MIC (2688 µM); where the statistical significance for HH427, HM605, and *Shigella* was P<0.0359, 0.0001 and 0.0058,

respectively. Therefore, testing had to be done whether geraniol inhibited bacterial growth under the same conditions of culture.

Figure 3. 5A illustrates that the MIC of wild type AIEC-HM605 was 2688 µM with *p*-value= 0.0003 in comparison to other concentrations. As DMSO was used as a solvent to dissolve geraniol in a concentration of less than 0.01%, there was a need to examine whether DMSO inhibited bacterial growth. Figure 3. 5B illustrates that the MIC for DMSO was greater than 2688 µM. Thus, the results shown in Fig 3. 5A reflect the effect of geraniol but not that of DMSO on bacterial growth.

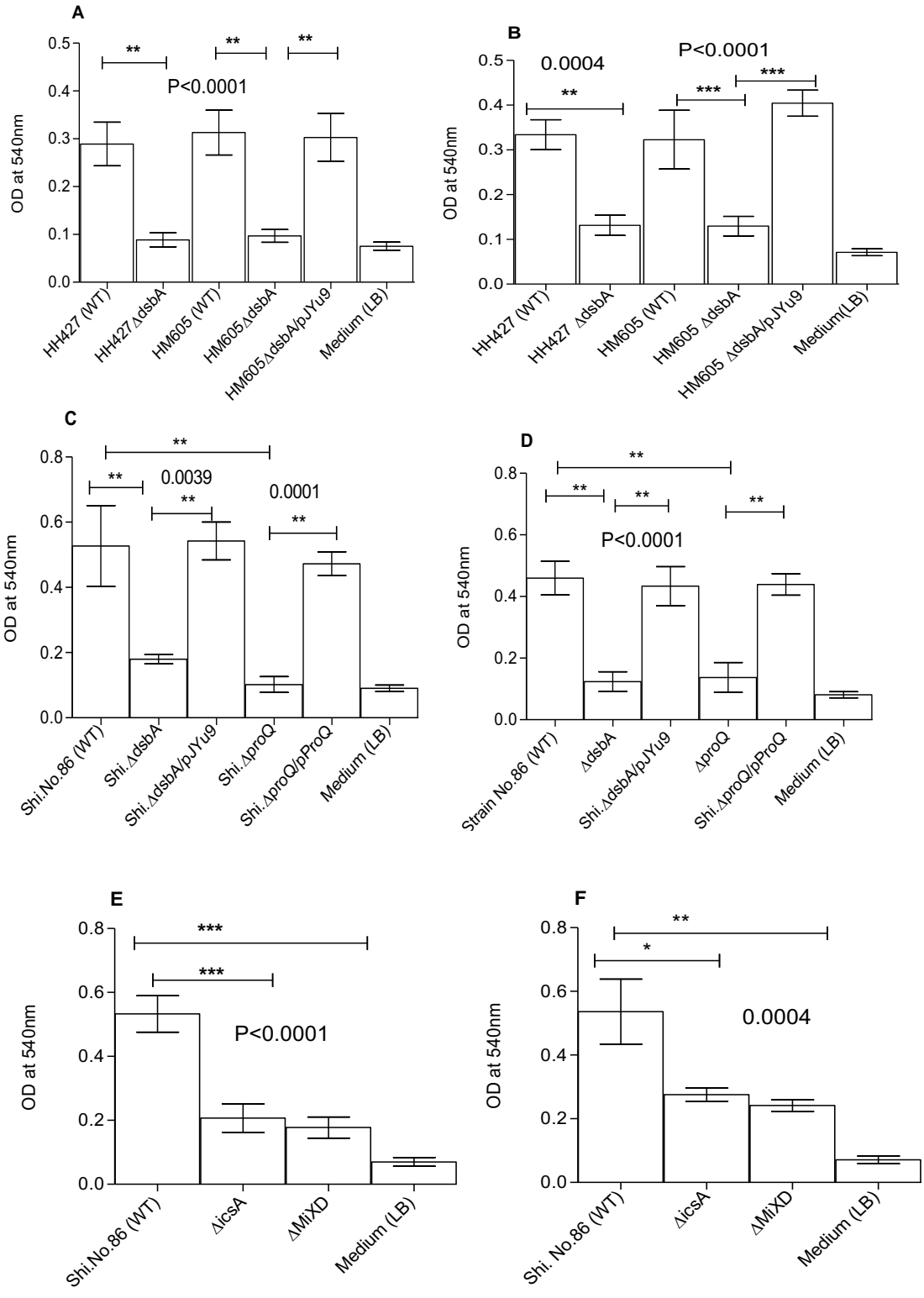


Figure 3. 3. Detection of Biofilms. Assays were carried out using 96-well polystyrene microtiter plates. Bacteria were grown at 37°C in LB broth containing bile salt (0.2% wt / vol)

for 24 h for (ACE) and 48 h for (BDF). Crystal violet was used to stain the biofilms. The tests were done in triplicate for each strain. Absorbance was measured at 540nm (A540) in a SoftMax Pro apparatus. Bars represent the mean value of the three readings. Error bars represent the standard deviation around the mean. Variation was significant (p <0.05) for *dsbA, proQ, icsA,* and *mixD* for *Shigella* and AIEC after 24 h or 48 h indicated by connecting horizontal bars.

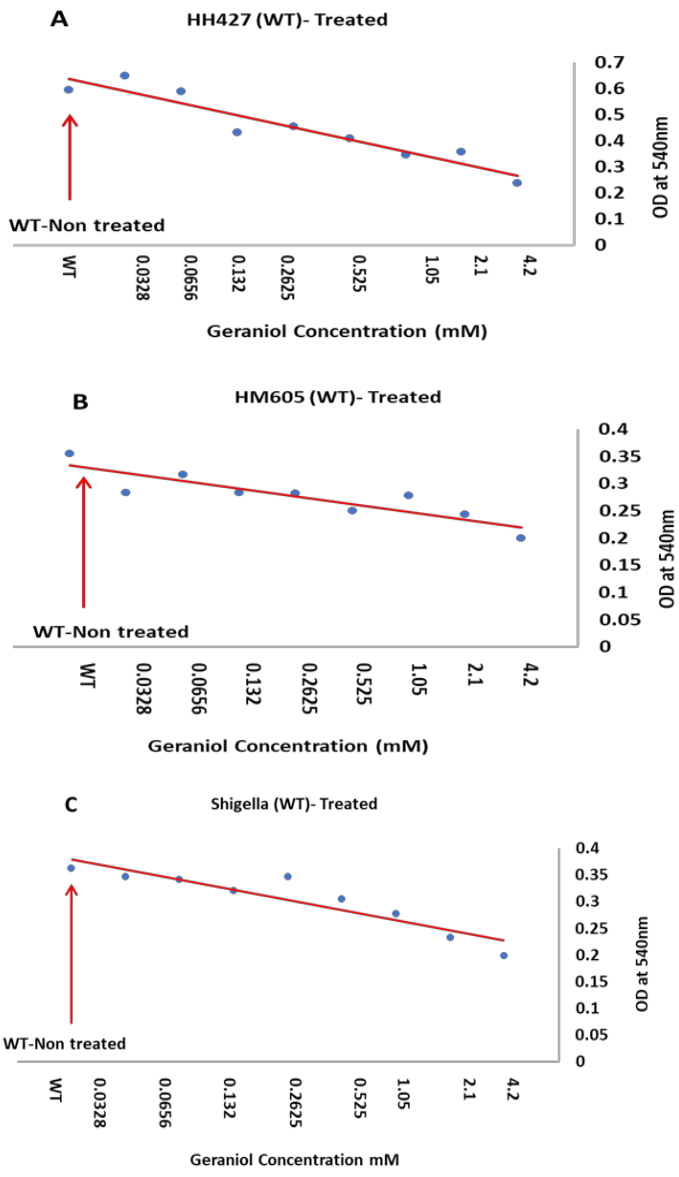


Figure 3. 4. Inhibition of biofilm by geraniol. The tests were carried out as in Fig. 3. 3 except that geraniol was supplemented in the medium during bacterial growth. The mean values of triplicate independent experiments are shown. Error bars show the mean standard deviation. X-axes depict geraniol-concentration and Y-axes depict absorption at OD540nm. Variation was found significant (P<0.0359, 0.0001 and 0.0058, for HH427, HM605, and *Shigella sonnei* respectively) between treatment with 4.2mM and untreated samples for all strains using Tukey's Multiple Comparison Test of one-way Anova GraphPad Prism 5 software.

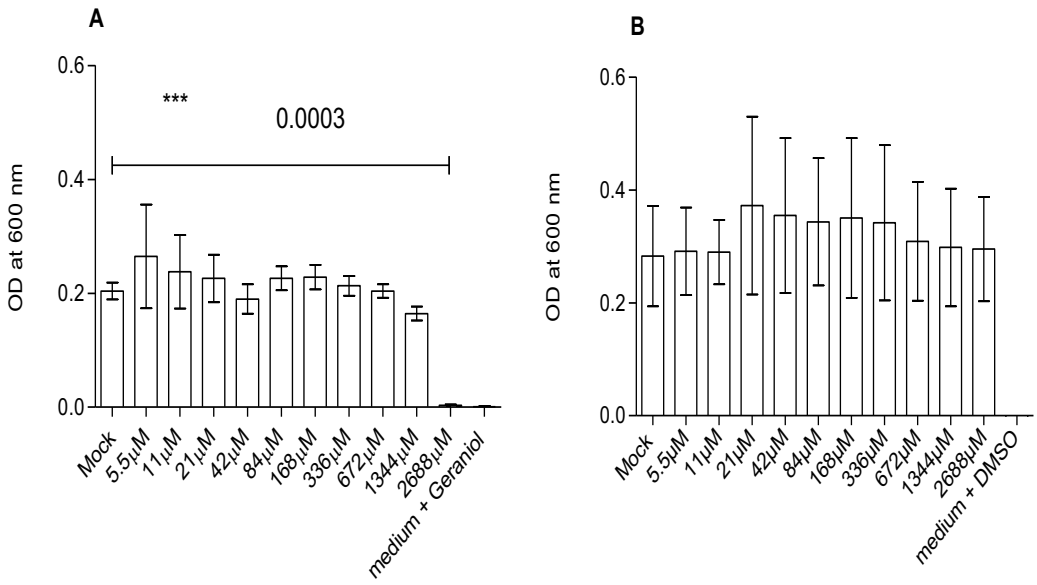


Figure 3. 5. Minimum inhibitory concentration (MIC) of geraniol (A) and DMSO alone (B) to AIEC strain HM605. Bacteria were cultured as in Fig. 3.3, geraniol and DMSO were added with indicated concentration using Muller-Hinton broth. OD600nm was taken after 24 h. The values are the means of three replicate determinations (n=3) ± standard error, pointed out that the MIC of geraniol is 2688µM with p <0.0003 in comparison to other concentrations indicated by horizontal bars connecting. The data were plotted using the bar graph functions in graph prism 5, where (X) represents geraniol or DMSO concentration, while (Y) represents the OD of bacterial growth.

3. 4. Discussion

3. 4. 1. Deletion mutants in *Shigella* and AIEC, and knockout's difficulties and mitigations in AIEC

In almost all infectious diseases, the ability to accurately classify the causative infection is a critical step in identifying or administering effective treatments. Microbial genetics and molecular cloning now allow us to study specific genes from a variety of microbial pathogens on a routine basis. Just as Koch's postulates, which describe the causal relationship between an organism and a particular disease, are formulated, it is evident that they are important in unravelling the potential role of a gene in infection and disease pathogenesis (Falkow, 1988). Thus, the deletion of the current genes in this study has a major impact on the experimental results in comparison to the wild type of the same background bacteria.

Changes to the original postulates of Koch justify infections caused by intracellular pathogens as well as the presence of pathogenic species types that are usually non-pathogenic. For example, *E. coli*'s predominant form is a member of the human intestine's normal microbiota, and it is generally considered harmless. Pathogenic strains such as enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (O157:H7) (EHEC) are present, but there is now substantial evidence that ETEC and EHEC exist as a result of the acquisition of new genes by the once- *E. coli*, which can produce toxins and cause disease. Minor genetic changes result in the pathogenic forms (Nina Parker, 2016). Here, Koch's molecular postulates have been successfully applied, and it demonstrates the important roles of these genes in biofilm formation. In addition, the importance of geraniol's antimicrobial activity against the production of biofilms from wild type strains used in this work.

According to, Hirayama *et al.* (2012) and Jost *et al.* (1997), counter-selectable double-crossover methods for the modification of bacterial genomes are sometimes difficult to make, and they are labour intensive. This stems from the low frequency of

recombination events. An alternative method of generating targeted generic modifications of both prokaryotes (Datsenko & Wanner, 2000; Yu *et al.*, 2000), and eukaryotes (Cherepanov & Wackernagel, 1995), which is fast, efficient and reliable, is the λ Red recombineering system (Murphy, 1998; Murphy *et al.*, 2000).

Different red λ plasmids were used in constructing deletions in *S. sonnei* and AIEC strains, due to their differences in resistance to ampicillin. The primers used for deleting the *S. sonnei dsbA* and *proQ* gene are standard, in that they generated 51 bp homologous extension flanking the kan cassette. This replaced the coding sequences of the *dsbA* and *proQ* genes, respectively. *S. sonnei dsbA* was deleted in previous work (Mahmoud *et al.*, 2016), and it was used in this study as a positive control. However, in order to replace the *dsbA* gene with the kan cassette in the AIEC strain, it was necessary to use phosphorylated primers, which harboured 71 bp homologous extension flanking the kan cassette. According to Maresca *et al.* (2010), using a phosphothionate at one end to protect from exonuclease degradation and the phosphorus on the other end at 5' will lead to facilitate the preferential generation of lagging-targeting primer (LSP) ssDNA cassettes at phosphothionate end and speed up exonuclease degradation of the unfavourable strand that as a result lead to increases the full-length, single-stranded, of LSP production. If both 5' ends are blocked to exonuclease, another activity (e.g. helicase) may separate the strands, thereby meaning that the full-length LSP can initiate recombination. However, due to the slow process of the supplier, Sigma, phosphothionate primers were not supplied. Fortunately, phosphorylated primers worked.

Another problem was that all AIEC strains were resistant to Ampicillin. For this reason, it was necessary to use pKDREG plasmid, which was resistant to chloramphenicol. In general, pKD46 with the Ampicillin marker appeared to be efficient for *Shigella* strains. Furthermore, the second cross over with plasmid pCP20 was problematic for AIEC because it was genuinely resistant to ampicillin. This is because the bacterium encodes beta-lactamase on the chromosomes of LF82 (UniProtKB), and HH427 and HM605. Therefore, transferring pCP20 (ampicillin

resistance) to these bacteria led to full bacterial growth on ampicillin-containing agar. However, individual true pCP20-containing colonies were obtained when bacteria were diluted after electroporation, from which *dsbA* deletion colonies were isolated after curing pCP20 at 42^{o}C.

An important clarification to make is that the pCP20 contains an ampicillin marker, which has a recognition site for the kanamycin cassette. This will lead to a connection and reaction with the kanamycin cassette, resulting in deletion regardless of the genuine ampicillin resistance. Further studies on this phenomenon would be recommended.

3. 4. 2. Knockout genes have not substantially affected growth-related bacterial fitness

The present work measured the growth rate of wild type and all mutant strains, and all strains grew equally well in L-broth. This excludes the growth deficiencies as an explanation for the different phenotypes of biofilm formation and invasion that were observed from the experiments in the following chapters.

3. 4. 3. Knockout genes have impaired the development of biofilms

A strong biofilm defect was identified (8.5% of wild type) with the *dsbA* mutant. However, an early study showed that *dsbB* disruption contributed only to a mild reduction (50%) (Niba *et al.*, 2007). It has been shown previously that *dsbA* has a significant role in *S. marcescens* (specifically, it encodes two homologues of *dsbA*, namely *dsbA*1 and 2). In this context, it is required for motility, virulence, and antibacterial activity in T6SS-mediated phenotypes. In T6SS-mediated antibacterial

behaviour, *dsbA* was found to play a significant role in both the proper functioning of the machinery in the secreting cell and the activation of the effector in the target cell (Mariano *et al.*, 2018). Where it was found that the deletion of the *dsbA* gene leads to a marked reduction in the number of assembled T6SSs and results in a significant reduction in an antibacterial activity dependent on T6SS due to the loss of core membrane protein stability TssM (Brunet *et al.*, 2015; Durand *et al.*, 2015). TssM is an integral membrane protein that constitutes an essential part of the T6SS core membrane complex.

Studies have found that loss of *dsbA* in *E. coli* has conferred partial resistance to *S. marcescens* ' T6SS considering inter-species interactions. However, given that resistance is incomplete, presumably due to the delivery of Rhs2 and other DsbA-independent effectors, the fitness consequences of DsbA loss on other cellular phenotypes are likely to outweigh that advantage. Thus, the researcher concluded that T6SS has the same action in AIEC regardless of the strain's variation. However, more studies should be conducted to identify the role of T6SS in AIEC (Mariano *et al.*, 2018).

Dailey and Berg (1993) observed that Dsb proteins are implicated in flagella assembly, and mutants are non-motile in the absence of cystine. *dsbA* mutants have a non-motile phenotype caused by the misfolding of the flagella motor P-ring protein in many bacteria, including non-pathogenic *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Dumoulin *et al.*, 2005; Hiniker *et al.*, 2005; Urban *et al.*, 2001). It is probable, according to Prigent-Combaret *et al.* (2000), that flagella are imported for the initial cell-to-surface contact, and thereafter the spread of bacteria along the surface. Evidence from prior studies indicates that there is reduced biofilm formation when there is an overproduction of several genes for flagella biosynthesis. This may result from the uncoordinated gene expression, which leads to the deficiency of flagella and reduced motility. The function of DsbA in adhesion is limited to the loss of flagella and type I pili. This is because forced contact between bacteria and cells, as well as the induced expression of type I pili, can restore the phenotype of the wild

(Bringer *et al.*, 2007). Thus, reduced biofilm formation of the *dsbA* mutant AIEC strains can presumably be attributed to impaired flagella and fimbriae formation.

Recent studies have shown that the composition of lipopolysaccharide (LPS) regulates the formation of biofilms in addition to the previously reported combination with variations in salt concentration, starvation or pH (Xu *et al.*, 2016). *dsbA* mutation of *shigella* will lead to firstly, inactivation of the *yihE* which plays an important role in LPS synthesis by regulating the *galETK* expression which determines *icsA's* surface expression. Secondly, the deletion of the *dsbA* gene will affect the *icsA* gene which is responsible for the motility resulting in the prevention of bacterial attachment to the surface which is the main stage in the formation of biofilms. LPS is a glycolipid located in the outer membrane of Gram-negative bacteria (Edwards-Jones *et al.*, 2004). Additionally, In the outer membrane (OM) the LPS assembly requires a properly folded LptD outer membrane protein. Its correct folding is SurA- and DsbA-dependent, which BepA can further fine-tune to remove misfolded LptD. LptD has two disulfide bridges between four non-consecutive cysteine residues requiring DsbA disulfide oxidoreductase periplasm and LptD's interaction with LptE to ensure correct folding with native disulfide bridges (Chng *et al.*, 2012). As a result, the mutation in *dsbA* gene will lead to inhibit biofilm production in both *Shigella* and AIEC.

Observations from this study encompass *Shigella* and additional enteric bacteria, including pathogenic strains of *E. coli*. Consequently, it shows that members of the Enterobacteriaceae family conserve bile salt-induced biofilm (Nickerson *et al.*, 2017). In this study, it was hypothesised that biofilm formation also promotes bacterial cell aggregation, thereby guaranteeing the successful transit of the small intestine for entry into the colon to cause infection (Nickerson *et al.*, 2017). However, the deletion of the abovementioned genes prevents biofilm formation in comparison to the wild type.

The amount of bile released into the intestine may change the colonisation of the gut. Low bile salt levels favour the proliferation of Gram-negative bacteria (including

pathogens), whereas high bile salt levels favour the proliferation of Gram-positive bacteria, as well as the reduction of Gram-negative bacteria (Bajaj *et al.*, 2012; Urdaneta & Casadesús, 2017). In the context, it is notable that the optimal conditions for intestinal bacterial strains are survival at pH 3.0 and bile concentrations of 0.3% (Kannan *et al.*, 2017; Usman & Hosono, 1999). This concentration of bile salt was approximately equal to the concentration used in the present work, namely 0.2% with no effect.

This study's results do not indicate biofilm stimulation in the presence of the concentrations of bile salts used for all mutated strains. This equates to the findings of other studies, where the physiological concentrations in the small intestine do not inhibit growth (Kannan *et al.*, 2017; Nickerson *et al.*, 2017). The *dsbA*, *proQ*, *icsA*, and *mxiD* mutants in *Shigella* were the most affected in biofilm formation (Figure 3.3A-F) when using LB broth containing bile salts, where it was found no effect of using bile salts in comparison to the wild type that produces biofilm similarly to the medium did not contain bile salts. Hence, the results suggest that biofilm inhibition resulted from the deletion of the above genes. This was consistent with other studies, where the physiological concentrations in the small intestine have not affected growth (Nickerson *et al.*, 2017). This is possibly related to the *Shigella* bile stress adaption.

In the context of biofilm formation, it is possible that *icsA* interbacterial self-association may contribute to bacterial aggregation (Koseoglu *et al.*, 2019). It was noted by Mauricio *et al.* (2017) that *icsA* has structural similarities when compared to autotransporter adhesins, including *E. coli* Ag43, which mediates aggregation and biofilm formation through self-association (Klemm *et al.*, 2006).

The *Shigella flexneri* protein IcsA has recently been shown to play a dual role in actin-based motility and adhesion, and its function as an adhesive, which is also TTSS-dependent, is essential for pathogenesis. The relationship between IcsA-dependent adhesion and T3SS behaviour in *S. flexneri* is supported by three experiments. First, IcsA is needed for hyper adhesion by the *ΔipaD* mutant, which possesses a constitutively active T3SS. Second, the elimination of needles in the *ΔipaD*

background removes hyper adhesion based on IcsA. Third, bile salt induces IcsA-dependent adhesion and invasion in wild type *S. flexneri*, whereas a needleless *Shigella* mutant does not respond to bile salt (Brotcke Zumsteg *et al.*, 2014; Mahmoud *et al.*, 2016). Therefore, the deletion of this gene leads to an inability to move, as a result of which no adhesion occurs, which leads to biofilm inhibition.

It was further noted by Koseoglu *et al.* (2019) that it is also is distinguished from these adhesins by its requirement for bile salt exposure to stimulating robust biofilm formation. The exact mechanisms implicated in the promotion of the formation of IcsA-mediated biofilm in the presence of bile salts are outlined in the sense that deoxycholate (DOC) is a more active inducer of biofilms than CA (cholate). In the case of DOC, it displays higher hydrophobicity and a greater ability to lead to protein aggregation than CA (Cremers *et al.*, 2014). Therefore, the stimulating effect of bile salts on the formation of biofilms may be correlated with changes in the IcsA conformation. This is supported by Brotcke Zumsteg *et al.* (2014) previous work, which indicated that DOC exposure modulates IcsA's proteolysis by neutrophil elastase. These findings suggest that DOC can affect IcsA's susceptibility to proteases by modifying the IcsA conformation (Koseoglu *et al.*, 2019). The Tran Van Nhieu lab demonstrated that secretion of the translocon protein IpaC occurs at one pole of the bacterium (Jaumouille *et al.*, 2008). The study also confirmed that, prior to secretion, cytosolic IpaC accumulates at the same pole as IcsA. This indicates that the bacterium is conditioned to deliver the effector proteins to the pole adhering to host cells, thereby maximising effector delivery and invasion. No biofilm is produced in mutated (i.e. *icsA* & *mixD*) strains of *Shigella* after 48 hours incubation in media containing bile salt, which indicted that biofilm inhibition took place as a result of gene deletion. These genes have an effect on the virulence and pathogenesis of *Shigella*, which is consistent with the findings reported elsewhere in the literature (Brotcke Zumsteg *et al.*, 2014; Koseoglu *et al.*, 2019). MxiD is a secretory protein that forms the basic structure of T3SSs called secretin's, which multimerise into stacked outer membrane

rings (Schuch & Maurelli, 2001). The data, therefore, suggested that TTSS is required for biofilm formation (Allaoui *et al.*, 1993; Dumenil *et al.*, 1998).

ProQ plays two molecular roles: firstly, translational mRNA repression with a trans-encoded sRNA that requires ProQ to work (Smirnov *et al.*, 2017), a mechanism close to that of the FinO-like RocC protein in *Legionella pneumophila*'s sRNA-mediated competency control (Attaiech *et al.*, 2016); and secondly, by binding at their 3' ends, ProQ stabilises mRNAs, protecting them from degradation by exonuclease RNase II (Holmqvist *et al.*, 2018). Hfq and ProQ have been identified as targeting most pathogenicity-related mRNAs (Chao *et al.*, 2012; Holmqvist *et al.*, 2016; Sittka *et al.*, 2007). Notably, the same scenario can be expected to occur with *Shigella* due to the deleted *proQ* gene, which stems from its similarity with *Salmonella.*

3. 4. 4. Geraniol inhibited biofilms formation, its potential applications

Since geraniol inhibits DsbA enzymatic activity, and since the *dsbA* mutants are defective in forming biofilms, one possible mechanism is via inhibiting *dsbA* for both AIEC and *S. sonnei*. For *S. sonnei*, another mechanism could involve inhibition of *dsbA*, leading to impaired TTSS. This relies on the fact that Spa32 is dependent on DsbA. The biofilm formation experiments were carried out to investigate the impact of geraniol against a wild type of *Shigella* and AIEC (HH427 and HM605) in the presence or without deoxycholate to induce bacterial proliferation and foster biofilm formation. It was found that geraniol inhibits biofilm formation for all strains, and the biofilm biomass quantification assay was employed to evaluate the antibiofilm activity of geraniol. The inhibitory concentration was around 4.2mM, which is consistent with the literature (Kannappan *et al.*, 2017; Pontes *et al.*, 2019; Singh *et al.*, 2012; Subramenium *et al.*, 2015). This merit will be added as an important point to geraniol applications in therapy.

Quorum sensing (QS) is an extremely important communication system for microorganisms. Various mechanisms used by essential oils (EOs), including geraniol, can inhibit bacterial QS in Gram-negative bacteria via acylated homoserine lactones (AHL) synthesis. However, in contrast to most Gram-negative bacteria, the genome of *E. coli* and *Shigella* seems to lack LuxI synthesis, and therefore does not produce AI-1. Autoinducer (AI-1) molecules are LuxI-type protein synthesised, and they belong to the molecular class of acyl-homoserine lactones (AHLs). It does, however, have a LuxR homologue, namely Cell Division Suppressor Inhibitor A (SdiA) (Wang *et al.*, 1991). SdiA may respond to AHLs secreted by other species of bacteria (Hughes *et al.*, 2010b; Rossi *et al.*, 2018; Soares & Ahmer, 2011). Also, *Shigella* has VirB, a transcription factor that is essential for the expression of *Shigella* virulence and requires a functional Autoinducer 2 (AI-2) quorum-sensing system (Day & Maurelli, 2001).

The first step of the mechanism can block the "upstream" mechanism, while a second downstream mechanism can act to block AHL transportation and/or secretion (Nazzaro, 2019). If bacteria still manage to produce AHLs, and if these molecules are still transported and secreted outside, they could be "captured" by other EOs, or their components, including γ-aminobutyric acid (GABA) and Gallic acid compounds like epigallocatechin gallate, ellagic acid, and tannic acid, naturally produced by many plants, are capable of interfering specifically with AHL-mediated signalling by blocking AHL-mediated bacterial communication (Riedel *et al.*, 2006; Sarabhai *et al.*, 2013), thereby preventing bacterial cell-cell contact. Therefore, other EOs act by exhibiting an antagonistic action in relation to AHLs or by performing an inhibitory effect downstream of the binding of AHL receptors (Nazzaro *et al.*, 2013a). The versatility of action in EOs depends primarily on their chemical composition, as well as the presence of functional groups. The antimicrobial activity of EOs also stems from their concentration and composition. Depending on these considerations, EOs or their components may operate on one or more factors that affect the mechanisms of cell-cell interaction between bacteria in different ways. Many EOs, even at low

concentrations, are therefore capable of preventing the chemical action of those enzymes that are involved in the production of energy for the survival and development of bacteria, or even disaggregating and denaturing microbial proteins at higher concentrations (Nazzaro *et al.*, 2013b; Tiwari R 2016).

3. 4. 5. Intervention with geraniol in the formation of biofilms and MIC

Essential oils (EOs) in the planktonic forms of microbes are known not only to be active against but also to have an inhibitory effect on the more resistant biofilms. Of the 10 terpenes tested, including geraniol, biofilm development of a *Candida albicans* strain was significantly reduced, and biofilm inhibition was induced by more than 80% when tested at concentrations of 0.06% (Nazzaro, 2019).

In this study, as detailed in the microdilution microplate reader test, the minimum inhibitory concentration (MIC) was evaluated in synchronisation with (Geraniol inhibited biofilm formation, its potential applications) (see material and methods). The test medium was a cation-supplemented Mueller-Hinton broth supplied by Oxoid Ltd., Basingstoke, UK. This is the recommended reference medium, as noted by the guidelines of the National Committee for Clinical Laboratory Standards (Standard, 2003). The medium is commonly used for broth dilutions in laboratories worldwide. It facilitates the effective growth of the majority of undemanding pathogens, and it is usually low in antagonists.

The results of the microdilution tests of geraniol oil are presented in Figure 3.4A. In agreement with the findings of Suppakul *et al.* (2003) and Solórzano-Santos and Miranda-Novales (2012), geraniol demonstrated outstanding antimicrobial properties against adherent invasive *E. coli* with MIC 2688µM. Because DMSO was used as a solvent to dissolve geraniol at a concentration below 0.01%, it was necessary to examine whether DMSO inhibited bacterial growth. Figure 3.5B shows a MIC greater than 2688 µM for DMSO. The results shown in Fig 3.5A thus reflect

geraniol's effect on bacterial growth but not that of DMSO. After 48 hours of incubation, the geraniol was found without effect using single dose (unpublished data), which depends on compound stability, aroma, and interactions with other chemicals in the test (Calvo *et al.*, 2018; Dicko, 2012; Faleiro, 2011; Lahlou, 2004; Swamy *et al.*, 2016).

In conclusion, the aim of the experimental work presented in this chapter was to identify the minimum inhibitory concentration (MIC) of geraniol against adherent-invasive *E. coli* (AIEC) in a microplate reader, and also to assess its activity against the formation of biofilms for AIEC and *Shigella*.

In terms of the results presented throughout this chapter, the researcher first drew attention to the plasmids and primers used for several of the experiments, after which the reasons for using specific primers were identified, and the success of phosphorylated primers was discussed. Following this, it was noted that the growth rate of wild type and all mutant strains was equally effective in L-broth, and so knockout genes did not substantially affect growth-related bacterial fitness. The chapter then reported several results and discussed these based on similar findings reported elsewhere in the literature. For example, it was noted that knockout genes impaired bacterial biofilm development, and that, in light of the study's finding that T6SS has the same action in AIEC regardless of the strain's variation, further studies should be undertaken to identify the role of T6SS in AIEC.

The experimental data also indicate that similar to other research projects, biofilm inhibition in this study resulted from the deletion of *dsbA*, *proQ*, *icsA*, and *MxiD* mutants in *Shigella*. At the same time, the results demonstrated that various genes are implicated in the virulence and pathogenesis of *Shigella*.

Chapter Four. *In vitro* evaluation of virulence and potential drug intervention using cultured RAW cells

4. 1. Introduction

4. 1. 1. The gentamicin protection assay

Many bacterial pathogens have evolved mechanisms for infection and survival inside host cells. For obligatory intracellular pathogens such as *Chlamydia*, assessing intracellular survival is uncomplicated; extracellular *Chlamydia* effectively will not survive and simple washes with saline are sufficient to allow for the assessment of virulence (Kokes & Valdivia, 2015); (Zuck *et al.*, 2017). Additional procedures are, however, needed in the case of facultative intracellular bacteria because, after washes, extracellular bacteria remain capable of growth to destroy cell monolayers. Therefore, a gentamicin protection assay has been developed to facilitate the investigation of the various intracellular virulence mechanisms of facultative intracellular bacteria (Kaneko *et al.*, 2016; VanCleave *et al.*, 2017). Gentamicin is an aminoglycoside with poor cellular permeability due to a lack of solubility in lipids (Hand & King-Thompson, 1986). Hence, it is frequently used in the antibiotic protection assay to assess the intracellular survival of bacterial pathogens. However, there is significant evidence to suggest that antibiotics, previously believed to be excluded by the host cell, are capable of gradually entering and accumulating within the cell (Flannagan *et al.*, 2016; Hamrick *et al.*, 2003; Menashe *et al.*, 2008). Therefore, E Myrdal *et al.* (2005) argue that aminoglycosides potentially artificially affect the intracellular growth of cytoplasmic pathogens. This should be considered during the design of antibiotic protection assays for the examination of intracellular growth. Nevertheless, it is generally agreed that gentamicin has poor cell membrane penetration and the gentamicin protection assay is commonly used in most laboratories for investigating the mechanisms of intracellular survival of pathogens.

The use of murine macrophage cells is a good model for testing AIEC invasion *in vitro*. The rationale for this was highlighted via the isolation of AIEC in macrophages

localised in the lamina propria in granulomas, and also within mesenteric lymph nodes of CD patients (Lodes *et al.*, 2004; Ryan *et al.*, 2004). A number of studies have shown that AIEC strains isolated from ileal and colonic CD patients are capable of surviving and replicating within J774A.1 murine macrophages and primary human monocyte-derived macrophages (Darfeuille-Michaud *et al.*, 2004; Glasser *et al.*, 2001; Meconi *et al.*, 2007; Mpofu *et al.*, 2007; Subramanian *et al.*, 2003). Furthermore, by secreting a range of pro-inflammatory factors such as cytokines and nitric oxide (NO), macrophages play a central role in the development of various inflammatory conditions (Lee & Park, 2016). Zong *et al.* (2012) suggested that adjustment of the macrophage-mediated response could prove beneficial in the design of new therapeutic protocols aimed at manipulating inflammatory conditions. The pro-inflammatory enzyme iNOS produces NO in excess, which leads to the development of various conditions such as asthma, arthritis, multiple sclerosis, colitis, and neurodegenerative disorders, as well as benefitting tumour formation and transplant rejection of septic shock (Patel *et al.*, 1999). These considerations warrant the identification and research of a novel drug that can regulate NO production. It is also worth noting that earlier studies have shown that geraniol can modulate the host immune system to produce anti-inflammatory cytokines such as IL-10 (Murbach Teles Andrade *et al.*, 2014). It has also been shown that γ-interferon and TNF-α can induce NO synthesis (Davis *et al.*, 2000; Wang & Leigh, 2006).

4. 1. 2. Microscopic imaging of live/dead cells with MTT

Cell viability levels and/or rates of cell proliferation are good measures of cell health. Cell metabolism may be influenced by physical and chemical agents. Such agents can cause cell toxicity through various mechanisms such as cell membrane destruction, protein synthesis prevention, and enzymatic reaction induction/inhibition (Ishiyama *et al.*, 1996). To evaluate the cell death caused by these mechanisms, short-term

cytotoxicity, and cell viability assays should be inexpensive, accurate, and reproducible (Aslantürk, 2018). Quantification of viable cells was commonly carried out using a methyl thiazolyl diphenyl tetrazolium bromide (MTT) assay, which essentially measuring mitochondrial function. Taking advantage that viable cells are able to turn 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) to a coloured formazan (Ng *et al.*, 2015), MTT can be added at the end of the gentamicin protection assay to quantify the viable cells.

Live-cell fluorescence imaging is a very effective approach to studying complex cellular processes and events and results in high spatial and temporal resolution. Over the past several years, the development of more stable and lighter organic fluorophores, fluorescent proteins, and nanocrystals has extended the toolbox considerably for researchers exploring cell biology boundaries. In addition to fluorescent reagents, advancements in optics, sensor technology, computing power, and improved software tools have been incorporated into imaging systems such as the EVOS® FL Auto Imaging System which are more powerful and user-friendly than in previous times (Frigault *et al.*, 2009; Waters, 2013).

4. 1. 3. Mutant AIEC strains under investigation

The role of *dsbA* in the virulence of pathogenic *E. coli* has been previously reported. DsbA, a periplasmic oxidoreductase, has been demonstrated to be required for expressing flagella as well as the type 1 pili. As a result, removal of *dsbA* results in impaired adherence to host cells due to the loss of flagella and type 1 pili, which are required to enforce bacteria-cell contact. Artificially induction of type 1 pili restored the wild type phenotype (Bringer *et al.*, 2007).

DsbA is also essential for the survival of AIEC strain LF82 inside macrophages, regardless of the loss of flagella and type 1 pilus expression. Where experiments show that LF82-Δ*fliC* mutant did not impair the ability of macrophages to survive and replicate. The survival capacity of the LF82 *dsbA* mutant was at the best to the level of a non-pathogenic *E. coli* K-12 strain (Bringer *et al.*, 2007).

Researchers have also found that the expression of type 1 pilus is mediated by a process called phase variation, where the bacteria switch between piliated and non-piliated states under the control of a switch-invertible element located upstream of the pilus-encoding operon type 1. Where DsbA was found to be involved in post-translation modifications of fimbrial structures such as bundle-forming pili in enteropathogenic *E. coli,* Pap pili in uropathogenic *E. coli,* and toxin-coregulated pili in *Vibrio cholerae* (Yu & Kroll, 1999).

Transcription of the *dsbA* gene was upregulated when LF82 was grown *in vitro* in a nutrient-poor and acidic medium that partly mimicked conditions encountered in the phagolysosome, suggesting that *dsbA* is activated under phagolysosomal conditions (Bringer *et al.*, 2006b). Therefore, the dsbA oxidoreductase is essential for LF82 to resist killing in the hostile environment of the phagosome. The inability of intracellular LF82-Δ*dsbA* bacteria to survive in macrophages is caused by the absence of functional HtrA protease due to the absence of a disulfide bond in the HtrA periplasm protease resulting in an unfolded and unstable HtrA protein (Skorko-Glonek *et al.*, 2006). However, intramacrophagic bacteria are unable to replicate in the absence of HtrA, but are capable of survival; whereas, in the absence of DsbA, they cannot survive (Bringer *et al.*, 2005). The reason for this could be that unfolded proteins can accumulate in the periplasm in the absence of DsbA oxidoreductase, leading to cell death. In addition, DsbA contributes to posttranslational modification of specific virulence determinants involved in the survival of intramacrophagic bacteria in AIEC strain LF82. Thus, the HtrA protease is not folded in DsbA-negative bacteria and therefore cannot fulfill its function in removing irreversibly damaged or abnormal proteins from the cell envelope (Clausen *et al.*, 2002).

In the case of *S. sonnei*, DsbA catalyses the formation of oxidised glutathione, GSSG, from reduced form, GSH, in the host cell cytosol; reduction of glutathione (GSH) level is vital for *S. sonnei* to survival in this hostile reducing cellular environment (Mirza *et al.*, 2018). These differential roles of the AIEC and the *Shigella* DsbA in intracellular survival are of interest to compare. Geraniol is able to inhibit *S. sonnei* DsbA leading to reduce bacterial growth; it is of intriguing to investigate if geraniol may inhibit AIEC intracellular growth despite AIEC is not exposed to the reducing cell cytosol environment.

Additionally, a mutant *S. sonnei* strain, *ΔproQ,* was evaluated in this study. The importance of *proQ* in pathogenesis was previously described in chapter three. *S. sonnei proQ* is homologous to *Salmonella* and *E. coli* where it has been shown to be required for bacterial motility, chemotaxis, and virulence genes; the mutant strain also triggers altered MAPK signalling in the host cells (Gonzalez *et al.*, 2017; Westermann *et al.*, 2019). ProQ has recently been identified as a global RNA-binding protein that binds dozens of sRNAs and hundreds of mRNAs in multiple *proteobacteria*; this suggests that ProQ may serve as a widespread regulator for bacterial gene expression (Pandey *et al.*, 2019).

4. 1. 4. Reactions of NO production and L-NAME inhibition

NO is a small molecule with several biological functions based on the site of production. For example, neuron-generated NO acts as a neurotransmitter, while macrophage-generated NO acts as an antimicrobial agent in response to invading microbes (Rosselli *et al.*, 1998). NO can move through the membrane of the cell readily and exert its cell action. It is biosynthesised from L-arginine in macrophages by nitric oxide synthase (NOS) (Pekarova *et al.*, 2011).

L-arginine is an abundant amino acid synthesised from glutamine, glutamate, and proline through the intestinal-renal axis. L-arginine is not harmful to cells in body

fluids and plays a unique role in the maintenance of immune homeostasis (Flynn *et al.*, 2002; Pekarova *et al.*, 2011; Wu *et al.*, 2009). Without L-arginine or NOS cofactors, macrophage-isolated iNOS will become uncoupled. Uncoupled NOS results in molecular oxygen, instead of L-arginine, being redirected, resulting in O2 as well as Peroxynitrite (ONOO) production (Sullivan & Pollock, 2006). ONOO−, a short-lived oxidant, and strong cell death inducer have been shown to be responsible for vascular disease progression, ischemia-reperfusion injury, circulatory shock, and inflammation (Chait *et al.*, 2005; Victor *et al.*, 2004; Wang & Leigh, 2006). The diagram below, adapted from Stuehr (2004) illustrates the synthesis of nitric oxide from L-arginine in mammals. Where all NOSs metabolise L-arginine to L-citrulline and NO through two consecutive monoxygenations based on NADPH.

L-NAME occurs naturally in living organisms as it is a product of arginine-methylised protein degradation (Bedford & Clarke, 2009), where it was used as an antagonist of nitric oxide synthase (NOS) in many animal models, including rats, to construct 'NO-deficient hypertension'. Many clinical trials have also been conducted to test its therapeutic potential for the over-production of NO diseases (e.g. septic shock). L-NAME slowly releases NO from its guanidino nitro group. The nitro group may be oxidised to a higher-state species of nitrogen oxide, such as N_2O3 and N_2O4 (ON-NO3), which then decomposes to NO (Liu *et al.*, 2019).

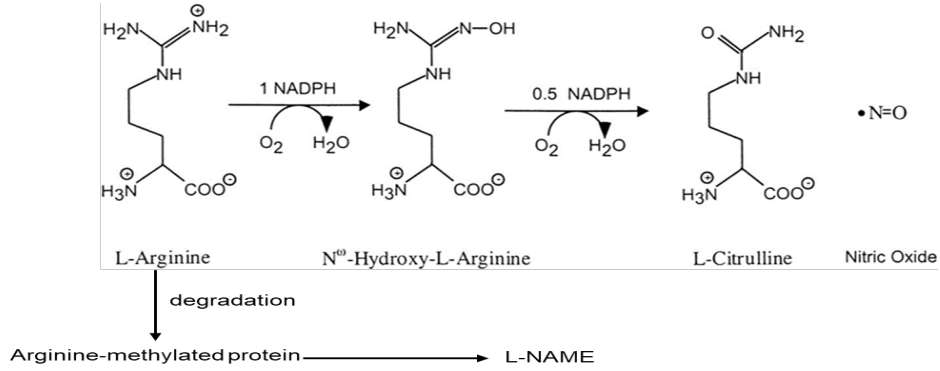


Figure 4.1. NO synthesis ' two reactions as catalysed by the NOS. The requirements for NADPH and oxygen for each reaction are indicated. L-name produced by the degradation of arginine-methylated proteins adapted from Stuehr (2004).

4. 1. 5. LPS as a standard agent that induces NO production

Bacterial LPS is well known as a mouse macrophage activation inducer, resulting in the production of NO, a radical gas with beneficial effects (e.g. microbial and tumour cell killing) and potentially harmful effects (e.g. tissue damage and septic shock) (Gao *et al.*, 1999; MacMicking *et al.*, 1997). The induction of NO secretion from mouse macrophages in response to LPS stimulation is due to increased expression of the inducible NO synthase (iNOS)[3] gene (Gao *et al.*, 1999). LPS stimulates three sub-classes of MAPKs (extracellular signal-regulated kinase (ERK), c-Jun-terminal kinase (JNK) and p38) in macrophages and other cell types. The figure below, adapted from Jamaati *et al*. explains how NO is produced to respond to bacterial infection.

Cytokine-inducible NOS (iNOS), which catalyses the synthesis of large quantities of NO from L-arginine and molecular oxygen, is primarily stimulated and regulated by a number of signalling pathways including the transcription factor of the nuclear factor-$_{\kappa}$B (NF-$_{\kappa}$B), and the protein kinases activated by mitogen (MAPK) (Yoon *et al.*, 2010).

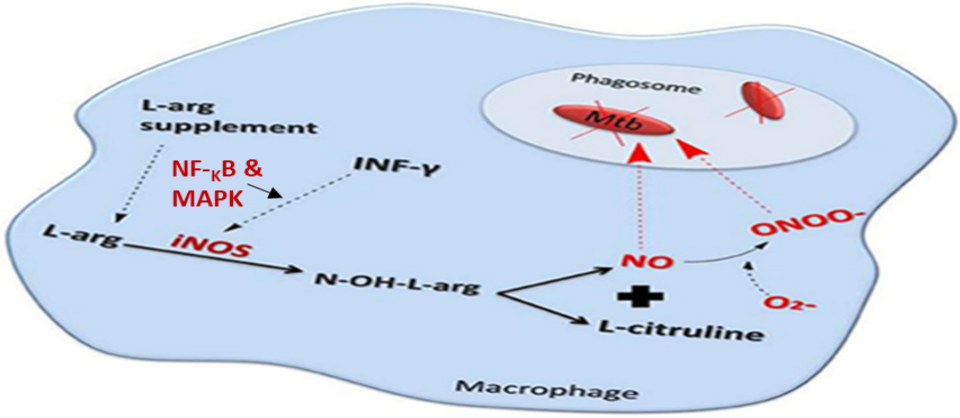


Figure 4. 2. The NO production pathway in macrophages. Interferon (IFN)-γ as well as other inflammatory stimuli, increase NO production by stimulating synthase (iNOS) of inducible nitric oxide. L-arginine also boosts NO production with elevated levels of the NO precursor.

To kill *mycobacteria* within the phagosome, NO may either act directly or in combination with superoxide (O2–) to form peroxynitrite (ONOO) adapted from (Jamaati *et al.*, 2017).

4. 2. Aims of the study

The study aimed to:

1. Evaluate virulence. The gentamicin protection assay was used to evaluate the invasion of mutants in macrophages.

2. Study the influence of geraniol viability and treatment compared to using L-NAME in NO production from RAW macrophages infected by AIEC.

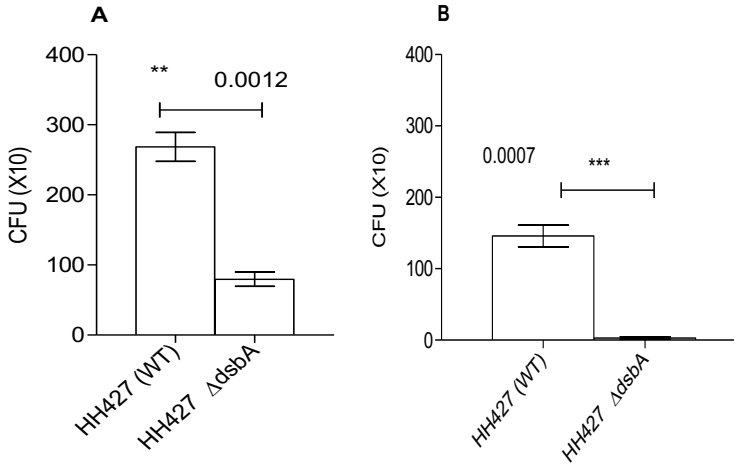
4. 3. Results

4. 3. 1. Gentamicin protection assay

A comparison of wild type AIEC and different mutants was made using a gentamicin protection assay with infection at MOI = 10 for 2 hrs or overnight. A notable difference was observed between the wild type strains HH427, HM605, and their respective *dsbA* mutants. The *ΔdsbA* mutants showed a significant drop in intracellular CFU 2 hrs post-infection (p = 0.0012 and p = 0.0115, respectively; Fig. 4. 3. A, C). Greater differences were observed between the wild type and mutants after overnight infection (p = 0.0007 and p = 0.0001, respectively; Figure 4. 3. B, D). When the mutant strains transformed with pJYu9 that contained intact *dsbA* gene the intracellular CFU was restored to the wild type levels (Fig. 4. 3. C, D). This suggests that the AIEC *ΔdsbA* was less capable of either invading or proliferating inside RAW macrophages.

The *ΔproQ* mutant of *S. sonnei* was also compared with its isogenic wild type using the same experimental conditions. A significant difference was observed between

the mutant and the wild type for 2hrs and overnight infection (p = 0.0005 and p = 0.0114, respectively) (Fig. 4. 3 E, F). Expressing *porQ* in trans restored the mutant to the wild type level.



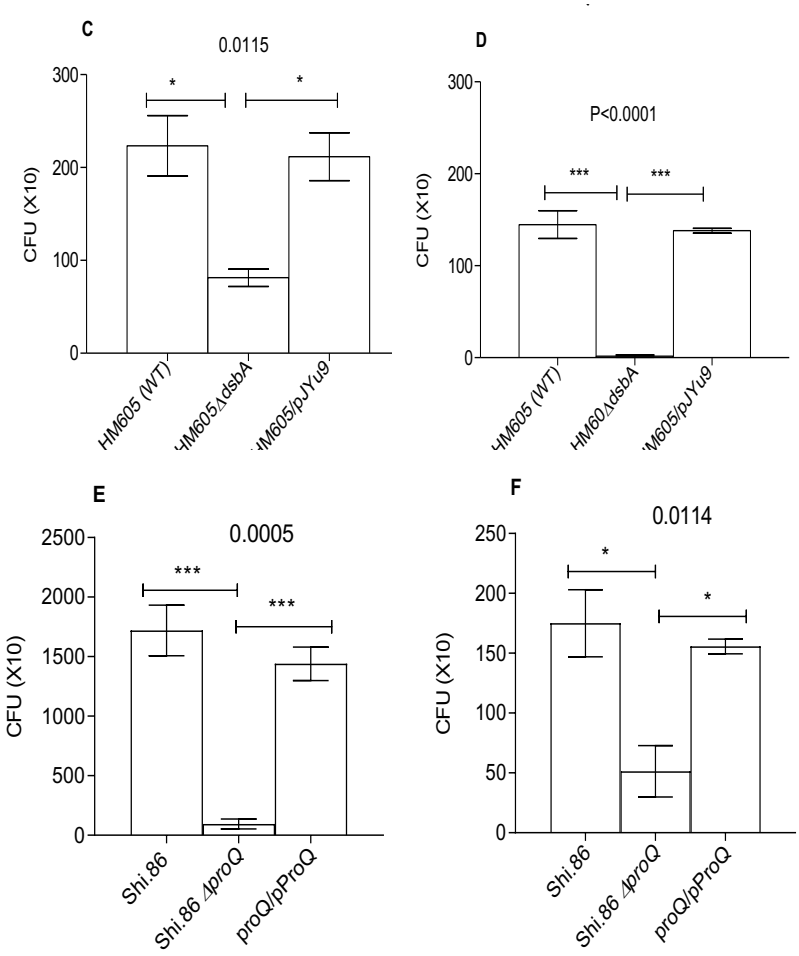


Figure 4. 3. Gentamicin protection assays. Infections of Raw cells were carried out with MOI = 10. Cells were lysed two h after infection (A, C, E) or overnight infection (B, D, F). Lysates were plated out on L-agar for colony forming units (CFU). Each value is the mean of three independent determinations ± SEM. Comparison between wild type and mutant strains was determined using Anova two way of prism 5. *p*-values are indicated by the horizontal link bars.

4. 3. 2. Live/dead cell microscopic imaging with MTT

In order to examine infected macrophages under EVO's microscope, all strains were transformed with pBS-ermGFP which constitutively expressed GFP. Twenty-four hrs post-infection, numerous wild type bacteria were observed within the RAW cells (Fig. 4. 4. A, C) whereas fewer *ΔdsbA* mutant bacteria were found within the cells (Fig. 4. 4. B, D). To correlate infection with cell death, MTT was added to the infected cells; viable cells converted MTT and became dark but dead cells remained colourless. The quantitation was done by counted 80 cells/wells under Evo's microscope and revealed that infection by wild type strains caused significant cell death compared to the cells infected with *ΔdsbA* mutant strains (Fig. 4. 5). The *p*-value was ≤ 0.0015 in both comparisons.

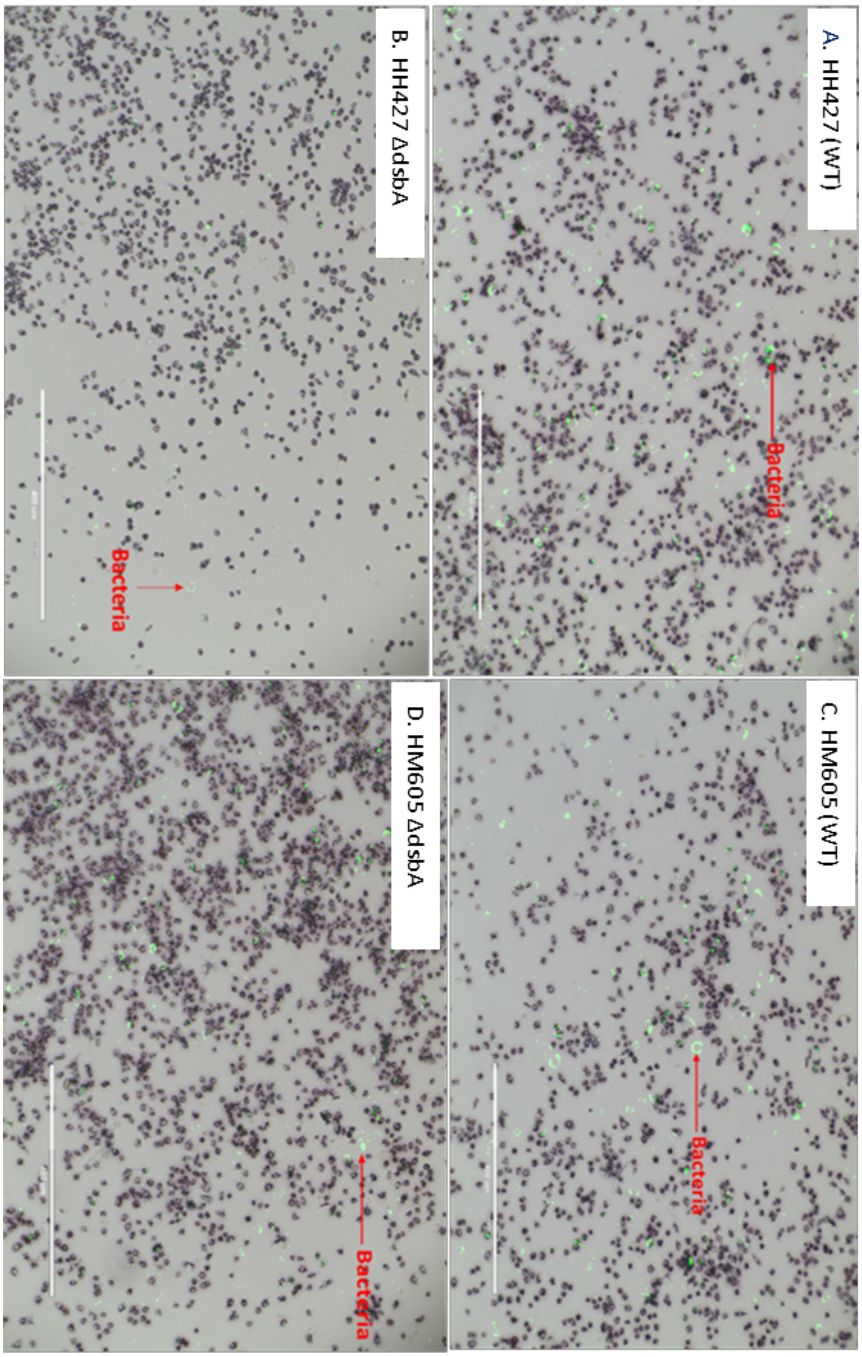


Figure 4. 4. Evos's microscopic examinations of Raw macrophages. Cells were infected with wild-type strains HH427 and HM605 (A, C), and their isogenic *dsbA* mutants (B, D). Bacteria were transformed with plasmid pBS-ermGFP for constitutive expression of GFP. The Raw cells were treated with MTT (0.5 mg/ml) one hour after infection; live cells form MTT-formazan

becoming dark whereas dead cells remained colour-less and overlap with green bacteria (arrows). Bars indicate 400 µm.

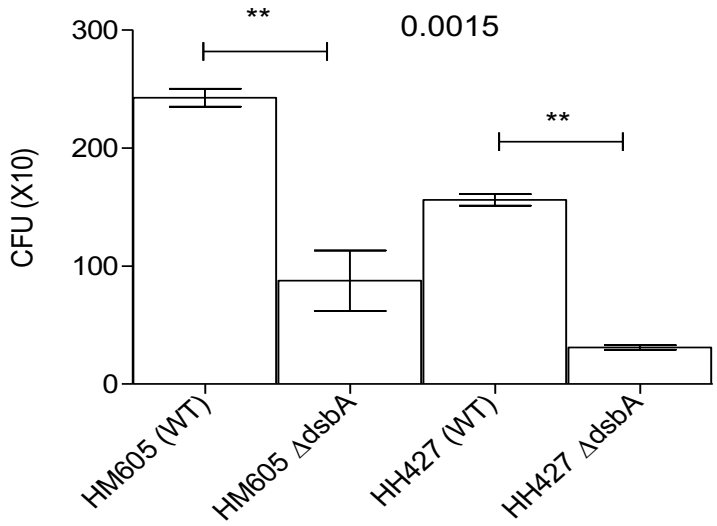


Figure 4. 5. Quantitative assessment of green fluorescent AIEC infected with raw macrophage. Each value is the mean of three independent determinations ± SEM. A significant difference was found between wild type and *dsbA* strains with *p*-value < 0.0015.

4. 3. 3. Inhibition of bacterial intracellular growth by geraniol

The impact of geraniol upon the intracellular AIEC was examined. RAW macrophages were infected with AIEC (HM605) at MOI = 10. In addition to gentamicin (50 µg/ml), the medium was supplemented with various concentrations of geraniol, and cells were incubated for 2hrs at 37°C under 5% CO_2. After 2 hrs of infection, the medium was removed, cells were washed three times with sterile PBS, and lysed with 0.1% (v/v) Triton. The intracellular CFU was numerated on lysates that were plated out on

L-agar. The intracellular CFU was reduced in the presence of geraniol in a dose-dependent manner and the IC_{50} was found to be 84 µM (* $p < 0.05$; Figure 4. 6). The intracellular CFU dropped further at 168 µM and higher doses (**$p < 0.001$). No significance was observed when using DMSO alone on bacterial growth (data not shown).

Viability was assessed using an MTT assay for geraniol and DMSO on the macrophage. The assay was performed to eliminate the possibility that the reduced intracellular CFU was due to the impact of geraniol to the host cells, which would allow gentamicin accumulating in the cells. DMSO was included in the assay as a solvent to dissolve geraniol in the stock solution. The results showed that RAW cells were tolerant of geraniol and DMSO, and at 84 µM (IC_{50} for geraniol) viable cells were comparable to the untreated controls (Figure 4. 7 A, B).

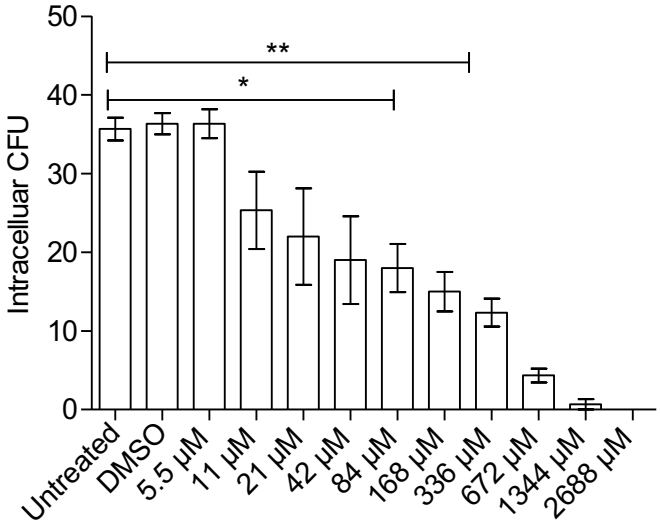


Figure 4. 6. Geraniol inhibition of intracellular growth of AIEC. Infection was done as in Fig. 4. 2 except that the cell culture medium was supplemented with geraniol at indicated concentrations. Mean values and standard deviation corresponding to the results of three separate experiments in triplicates. At 42 µM, the intracellular CFU reached a significant drop (*$p < 0.05$) and at 168 µM the intracellular CFU dropped further (**$p < 0.001$).

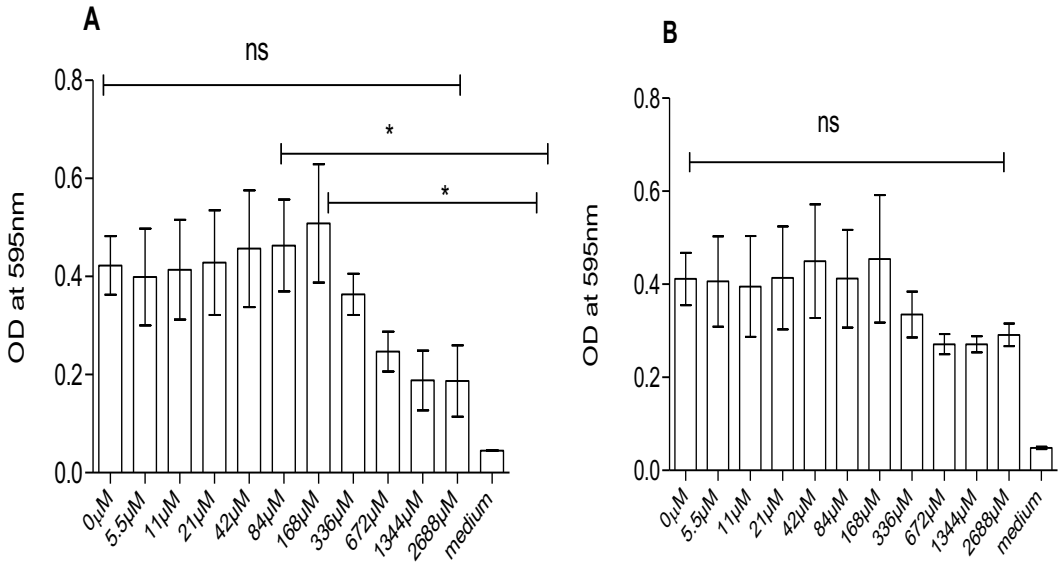


Figure 4. 7. MTT assay for viability. (A) geraniol in DMSO and (B) DMSO alone on RAW 264.7 macrophages. The Raw cells were cultured in DMEM containing geraniol or DMSO with indicated concentrations overnight under 5% CO_2 at 37°C. MTT assay was carried out as described in Material and Methods. The data are the averages of three separate experiments with triplicates; the error bars represent the standard deviations.

4. 3. 4. Nitric oxide production and inhibition

Concentrations of NO_2- in the culture medium were considered to be indicative of NO production (as described in the Methods section). This is directly indicated by the measurement of OD540nm (Fig. 4. 8 A). Infection of Raw cells by AIEC strain HH427 (MOI = 10) or treatment of Raw cells with LPS (1µg/ml) induced huge NO production compared to the control cells (**p < 0.01; Fig. 4. 8 A). The NO production was significantly reduced in the presence of 84 µM geraniol or 25 mM/ml L-NAME from AIEC-infected Raw cells (*p < 0.05). However, the NO production was only reduced in the presence of 25mM/ml L-NAME (*p < 0.05) from LPS-treated Raw cells; the presence of 84 µM geraniol had no influence on NO production from LPS-treated Raw cells. These data suggested that both infections by AIEC or treatment by LPS increased NOS activity, in turn, resulted in NO production. L-NMAE inhibited NOS activity, therefore, inhibited NO production from Raw cells infected by AIEC or treated by LPS. On the other hand, geraniol had no activity against NOS, therefore, it made no reduction of NO production from LPS-treated Raw cells. Geraniol, however, made a reduction of NO production from infected Raw cells due to its inhibition of intracellular AIEC bacteria.

To test the above hypothesis, the intracellular CFU was counted in the presence of geraniol or L-NMAE as in Fig. 4. 6. The results showed that the intracellular CFU were significantly lower from cells treated with geraniol than cells treated with L-NAME or non-treated infected Raw cells; there was a significant difference with a p-value of 0.0174 (Fig. 4. 8 B).

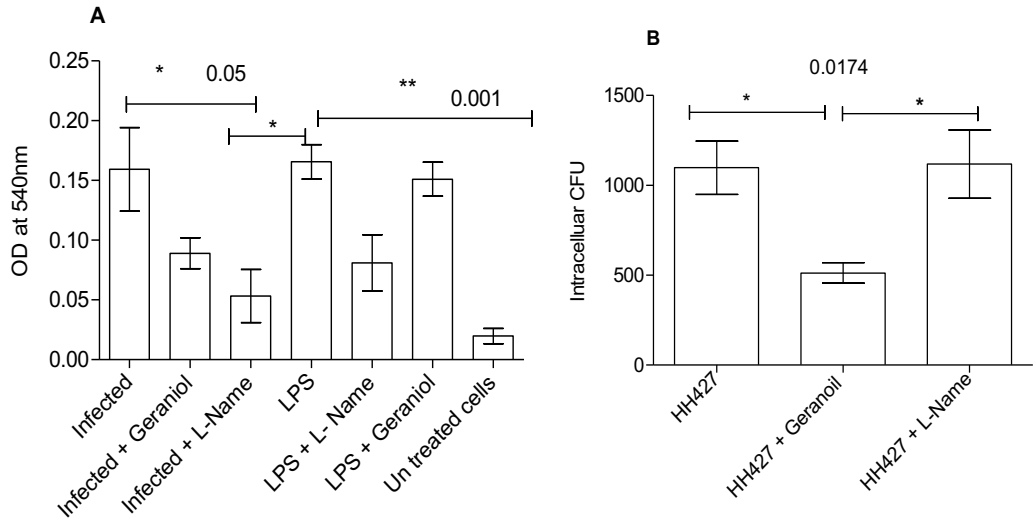


Figure 4. 8. A. NO production. RAW 264.7 cells were infected by HH427 (MOI = 10) or treated with 1 µg/mL of LPS overnight, with or without the presence of geraniol (84 µM/ml) or L-NAME (25 µM/ml). Means and standard deviations were calculated from three independent experiments with triplicates. Asterisks * and ** indicate $p < 0.05$ and $p < 0.001$, respectively. **B. Impact of geraniol or L-NAME on the intracellular CFU.** Experiments were carried out as in Fig, 4.6, and combined data from three independent experiments were plotted. The asterisk indicates significance between groups of experiments ($p < 0.0174$).

4. 4. Discussion

4. 4. 1. 1. Possible mechanisms for AIEC as a result of *dsbA* deletion

In order to establish the role of *dsbA* in the virulence of the AIEC strains (HH427 & HM605), mutants with the *dsbA* gene deletion were constructed (Fig. 3. 1. B). It is apparent that the HH427 & HM605-*ΔdsbA* mutants produced significantly reduced intracellular CFU 2 h and overnight post-infection (Fig. 4. 3). This is consistent with previous reports about strain LF82; removal of *dsbA* resulted in an inability of replication inside of the host cells (Bringer *et al.*, 2007). In this regard, intracellular replication as a result of *dsbA* deletion may impact on more intracellular survival than the strain's ability to adhere to or invade host cells, albeit deletion of *dsbA* also impaired flagella and type I pili which are important for adherence (Brien *et al.*, 2017). The fact that complementation with pJYU9 restored intracellular CFU 2 h and overnight post-infection demonstrated that the deletion of *dsbA* alone was responsible for the failure of intracellular survival and proliferation. Once again, this demonstrated the usefulness of the molecular Koch's postulation in assessing the role of the individual gene in a particular phenotype, in this case, the bacterial virulence.

From this study's literature, particularly the discussion of AIEC pathogenesis given in Chapter 1, one of the possible steps in the pathogenesis of AIEC involves employing OmpA that has many diverse roles in adhesion, invasion, and persistence of intracellular bacteria (Nicholson *et al.*, 2009). OmpA requires DsbA for correct folding (Smith *et al.*, 2016). In the null *dsbA* background, OmpA won't fold properly, which may directly impact adherence and invasion and as a result account for the low number of bacteria inside the macrophages. The inability of intracellular LF82-*ΔdsbA* bacteria to survive in macrophages is caused by the lack of functional HtrA protease due to the absence of a disulfide bond in the HtrA periplasm protease which results in an unfolded and unstable HtrA protein (Skorko-Glonek *et al.*, 2006).

4. 4. 1. 2. Live/dead cell microscopic imaging with MTT

It should be stressed that MTT assay is suitably applied to purposes such as screening cytopathogenicity in macrophages and is preferable to other tests because of its simplicity and reproducibility (Ebtekar *et al.*, 2006; Sudo *et al.*, 1994).

Using the same criteria in MTT staining of the epithelial cells and macrophage by (Ebtekar *et al.*, 2006; Grabinger *et al.*, 2014) the above results (Figure 4.3) were reinforced by examining infected macrophages under EVO's microscope, where all strains were transformed by using pBS-ermGFP which constitutively expressed GFP. Numerous wild-type bacteria were observed in the RAW cells twenty-four hours after infection, whereas fewer mutant bacteria were found in the cells (Fig. 4. 4. E, G vs. Fig. 4. 4. F, H). MTT has been added to the infected cells as one of the most delicate tests in this respect (Ebtekar *et al.*, 2006); viable cells have converted MTT and have become dark and dead cells remained colourless. This was supported by counted the infected cells under Evo's microscope and showed that wild-type strain infection caused significant cell death compared to cells infected with mutant strains (Fig. 4.5). The circular, green coloured bacteria were counted and considered as one colony under the microscope, so the enumerated high number reflects the number of bacterial invasions in the macrophage. In metabolically active cells, the tetrazolium salt MTT is reduced to water-insoluble violet formazan crystal by means of mitochondrial dehydrogenases, predominantly succinate dehydrogenase (Ng *et al.*, 2015; Peng *et al.*, 2005), which give a viable macrophage the dark colour, whereas non-viable has not the enzyme mentioned above, thus appearing colourless or cavity containing green-coloured bacterial colonies.

4. 4. 1. 3. Possible mechanisms for *S. sonnei* as a result of *proQ* deletion

In the present study, the model of Raw cell infection was used to determine the role of *proQ* deletions in *S. sonnei*. Wild type *S. sonnei* infection with raw macrophages was found to have resulted in a high number of overnight bacterial intracellular proliferation with a low number of macrophages, equivalent to a 2-h reduction. In contrast, a small number of intracellular bacteria were generated by the *proQ* mutant, suggesting that this gene plays a major role in intracellular survival as well. ProQ-dependent gene expression was found to change when human cells become infected with *Salmonella*. Such modifications include dysregulation of the genes of bacterial motility, chemotaxis, and virulence, followed by altered MAPK (mitogen-activated protein kinase) in the host (Westermann *et al.*, 2019). The deletion of *proQ* results in weaker MAPK signals compared to wild type *Salmonella*. The MAPK signalling cascade is essential for activating the responses to the nuclear factor and interferon. It is one of the primary targets of bacterial pathogens since they manipulate immunity for their benefit (Arthur & Ley, 2013). For example, specific effectors of the SPI-2 regulon, SptP, whose expression in *Salmonella* bacteria increases in the null *proQ* background. As a result, the MAPK singling is inhibited by SptP inhibition of extracellular signal kinase (Mazurkiewicz *et al.*, 2008; Odendall *et al.*, 2012).

ProQ is critical to directly binding to individual SPI-1 transcripts of structural components of the SPI-1 needle or secreted effectors (e.g. binding of ProQ to SicP-SptP, a transcription unit which is important SPI-1 effector SptP and cognate *SicP* chaperone) (Holmqvist *et al.*, 2018; Mazurkiewicz *et al.*, 2008). ProQ targets transcripts of both the *prgHIJK* and *sicP*- controlled SPI-1 operations. Studies found that deleting *proQ* reduced the steady- levels of *prgI* mRNA while improving the expression of *sptP*. The increase of SPI-1 effector level SptP leads to inhibition of MAPK cascade by ERK (extracellular signal-regulated kinase) (Lin *et al.*, 2003). As a result, in the absence of the RNA chaperones (ProQ regulons), key virulence systems,

such as the flagellum or the SPI-1 secretion system, were dysregulated (Mazurkiewicz *et al.*, 2008).

The study also revealed virulence attenuation, which resulted in a decrease in the bacterial invasion to raw macrophages, compared to wild type after 2 and 24 hrs of post-infection in gentamicin protection assay, linking these results to the deletion of *proQ* in *Shigella* (Fig. 4. 2. E-F). *Shigella* secretes effectors via the *mxi-spa* T3SS, to enforce invasion. Thereafter, *Shigella* quickly escapes from the phagosome, thus avoiding phagosome-lysosome fusion and degradation. Instead, the bacteria kill the macrophage once they have escaped from the phagosome by IpaB (Clerc *et al.*, 1987; Hilbi *et al.*, 1997; Hilbi *et al.*, 1998). According to Hilbi *et al.* (1998) and Chen *et al.* (1996), macrophage death is mediated by the secreted translocator/effector protein IpaB. The nucleotide sequence analysis of the genes encoding two of the *Salmonella typhimurium*, namely the SipB and SipC secreted proteins, revealed that they are homologous to the IpaB and IpaC *Shigella* invasions, respectively. SipB and SipC are the parts that composed the SPI-1 effectors (Kaniga *et al.*, 1995; Wang *et al.*, 2017). Therefore, based on the above, as well as in view of the similarity between *Shigella* and *Salmonella*, it seems reasonable to conclude that a similar action would occur in *Shigella* as well.

Another finding reported by Chaulk *et al.* (2011) indicated that *proP* expression levels are downregulated when *proQ* is removed. The *proP* gene in *E. coli* encodes a low-affinity osmoprotectant transporter, like proline and glycine betaine, which senses extracellular osmotic pressure and responds by maintaining membrane turgor pressure (Wood, 2006). *proP* is a superfamily member of the main facilitator, and it is classified as an osmoprotectant proton symporter controlled by high osmotic pressure (Lim & Lee, 2015). *ProQ* lesions encoding RNA chaperone result in the decreased ProP protein level of expression (Kerr *et al.*, 2014). Hence, a possible scenario is that *proQ* deletion led to impaired ProP, which in turn contributed to osmotic intolerance in the phagolysosomes. Finally, this could have resulted in the impaired intracellular proliferation of *Shigella*.

4. 4. 2. Inhibition of bacterial intracellular growth by geraniol

Macrophages function to engulf bacteria and contain them within the phagosome, which fuse with lysosomes leading to the degradation of the bacteria. AIEC can, however, escape the cycle of degradation and develop within phagolysosomes (Flanagan *et al.*, 2015). This is because AIEC can live in the acidic, nutrient-poor environment of the phagolysosome (Bringer *et al.*, 2006a). Due to the previous finding that geraniol inhibited *S. sonnei* intracellular growth, here geraniol was therefore investigated as to whether inhibition also occurs to AIEC inside Raw cells albeit AIEC lives in phagolysosomes whereas *S. sonnei* lives in cells cytosol.

It was found that 84 µM geraniol significantly reduced intracellular CFU 2 h post-infection (Fig. 4. 6. A). The results were consistent with those reported in a number of previous studies (Juárez *et al.*, 2016; Lapczynski *et al.*, 2008; Tiwari & Kakkar, 2009). Figure 4. 6A indicates that there was a significant difference between the dose used in this study (84 µM) and 5.5 µM, with $p < 0.05$, when compared to the minimum inhibitory concentration outside and inside the macrophage. In addition, the use of this dose does not affect the viability of the cell line used in this study, such as macrophage, and organoids infection. Where the MTT on the macrophage was used to exclude effect of geraniol and DMSO (Figure 4. 7 AB). The test helped eliminate the possibility that the reduced intracellular CFU was due to the cytotoxicity of geraniol to the host cells, allowing gentamicin to accumulate in the cells. The results showed that there was no effect on the RAW cell treated with the different geraniol and DMSO concentrations. DMSO was included in the assay as a solvent for dissolving geraniol in-stock solution (Figure 4. 7 B).

Although DsbA plays a key role in bacterial growth in acidic, nutrient-poor environments, the probable mechanism for geraniol to reduce intracellular CFU may be that it can destroy AIEC in phagocytic vacuoles by inhibiting *dsbA's* activity. It is unlikely that geraniol can target intracellular AIEC - DsbA in the same way as it does to intracellular *S. sonnei* DsbA. Geraniol competitively inhibits *Shigella* DsbA by

competing with glutathione (GSH) for the binding active site (Mirza *et al.*, 2018). GSH is present in cell cytosol within 1–10 mM range about 10-fold over Glutathione disulfide (GSSG) (Meister, 1988). However, the phagolysosomes in which AIEC resides are unlikely to have the same GSH/GSSG ratio. Previous studies show that HtrA/DegP protein is dependent on DsbA for its activity as a protease (Skorko-Glonek *et al.*, 2008). Furthermore, HtrA/DegP has been shown to be vital for AIEC survival in the phagolysosomes (Bringer *et al.*, 2005). Hence, binding of geraniol to the active site of DsbA in the phagolysosomes would deprive the internal disulphide bond of HtrA, in turn resulting in the inability of the mutant to cope with the acidic environment stress.

4. 4. 3. Geraniol as potent in inhibition of NO production via inhibition of intracellular bacterial proliferation

This work investigated whether geraniol activated and protected macrophages against bacterial infection or LPS treatment. The results revealed that infection by both AIEC and *S. sonnei* can cause NO production. Both L-NAME and geraniol inhibited NO production in infected macrophages. However, the mechanisms differ: L-NAME inhibits nitric oxide synthase (NOS) whereas geraniol inhibits bacterial intracellular growth (figure 4. 8 A). However, both Medicherla *et al.* (2015) and Su *et al.* (2010) reported inconsistent results in finding that geraniol led to a significant reduction in NO production by modulation of iNOS enzyme. The results in Figure 4.8A confirmed that geraniol caused intracellular CFU reduction, in turn, resulted in reduced NO production. In comparison to L-NAME treatment which did not cause a reduction of intracellular CFU; the observed NO reduction was presumably due to the direct inhibition of NOS by L-NAME.

A study on mouse models, by Stuehr and Marletta (1985), showed that NO^{2-} demonstrated toxicity against bacteria and that there was a close association between the occurrence of NO^{2-}/NO^{3-} production in Bacillus Calmette-Guérin vaccine (BCG) infection and the development of increased non-specific bacterial resistance. Meanwhile, Hibbs *et al.* (1987) argued that toxicity to neoplastic cells when cultured alongside activated macrophages might be mediated by NO^{2-}/NO^{3-} synthesis.

To sum up, the experimental findings indicated that all knockout mutants were attenuated in intracellular proliferation. Furthermore, *proQ*-dependent gene expression was found to change when human cells were infected with *Salmonella,* which the researcher posited would be similar in the case of *S. sonnei.*

The chapter also discussed the implications of the results, obtained from a gentamicin-killing study, that geraniol inhibited bacterial intracellular growth. Similar findings reported elsewhere in the literature were drawn on to examine the experimental findings, where the purpose of the experiment was to determine whether geraniol inhibits AIEC growth in RAW 264 mouse macrophages.

Finally, geraniol's status as a potent inhibitor of NO production, as well as the mechanism of action, in this case, was investigated. both geraniol and L-NAME inhibited NO production in infected macrophages (albeit by contrasting mechanisms). It is worth emphasising that, throughout the chapter, all of the above results were consistent with the results regarding biofilm formation, larval, and organoid infections. In the next chapter, the experimental foundation set in both this chapter and the preceding chapter is extended to assess the virulence of AIEC and *Shigella* using the *Galleria* larvae model.

Chapter Five. *In vivo* evaluation of virulence using a *Galleria* moth larvae model

5. 1. Introduction

Insect model organisms are inexpensive, widely available, and do not present militant ethical issues. The *Galleria mellonella* larvae model has been used for evaluation of the microbial virulence as well as therapeutic agents (Hamamoto *et al.*, 2004). According to Barnoy *et al.* (2017), there is a reputed similarity between the intestinal cells of the mammalian digestive system and that of the insect larval midgut. Testing the hypothesis of physiopathology is possible (Lavine & Strand, 2002) because of the similarities of insect and human immune systems. Specialised cells, called haemocytes, phagocytose pathogens forming aggregates that encapsulate and neutralise foreign microorganisms, which take place in most insects (Ribeiro & Brehelin, 2006). In addition, a phenoloxidase (PO) melanisation cascade can be activated by haemocytes (Lavine & Strand, 2002). In response to bacterial infection, haemocytes produce and secrete antimicrobial peptides (AMPs) by the activation of specific signalling pathways, including Defensin and Defensin-like peptides such as Defensin, Drosomycin, Holitricin, and Sapecin. Such peptides are rich in cysteines and contain cysteine disulphide bonds (Hillyer, 2016; Ribeiro & Brehelin, 2006). The insect fat body, an organ similar to the mammalian liver, may generate reactive oxygen species, which also kill invading organisms.

5. 1. 1. Genomic/transcriptomic relevance of *Galleria* larvae

The wax moth larva of *Galleria mellonella* has been used as a model for investigating infections caused by a variety of pathogens, including a number of gastrointestinal bacterial pathogens (Mahmoud *et al.*, 2016; Ramarao *et al.*, 2012; Tsai *et al.*, 2016; Viegas *et al.*, 2013). This *in vivo* model has several practical advantages, for example,

it is feasible for conducting a high number of replicates per test and presents ease of management for test conditions, larvae are small, simple to handle, and inexpensive. The *in vivo* model has also been used for a number of other tests including antifungal, antibiotic, and phage treatments (Abbasifar *et al.*, 2014; Benthall *et al.*, 2015; Lionakis, 2011; Mesa-Arango *et al.*, 2013). Additionally, the complete transcriptome of *Galleria* is available (Vogel *et al.*, 2011) in response to bacterial lipopolysaccharide (LPS). Injection of LPS provoked powerful immune responses from *Galleria* larvae (Altincicek & Vilcinskas, 2007a; Altincicek & Vilcinskas, 2007b; Seitz *et al.*, 2003).

Two LPS-binding proteins were identified in *Galleria mellonella* which are considered microbe-associated molecular patterns (PRRs) (Brivio *et al.*, 2005). A highly abundant PRR is apolipophorin in lepidopteran insects which binds to LPS and triggers antimicrobial responses (Brivio *et al.*, 2005; Staczek *et al.*, 2018). Studies have found that *Galleria mellonella* is able to induce immune responses to different microbes with varying intensities, which also assumes differential recognition (Mak *et al.*, 2010). Interestingly, the upregulated expression of the hemolin gene, which is abundant in insect haemolymph and interacts with LPS and lipoteichoic acid (LTA) was also found (Yu & Kanost, 2002). Hemolin, a member of the superfamily of immunoglobulin, is also proposed to function as a molecule for recognition. Apolipophorin and hemolin both work as opsonins (Tsai *et al.*, 2016; Yu & Kanost, 2002).

Insects and mammals share similar effectors that are secreted to microbial challenges, such as reactive oxygen species (ROS)/reactive nitrogen species (RNS) production or antimicrobial peptides (AMPs) (Kim & Lee, 2014; Xiao *et al.*, 2017). *G. mellonella* induces ROS- and RNS-related responses by *nox-4* and *nos* expression, which together with antimicrobial peptides like gallerimycin a defensin-like antimicrobial peptide (AMP) that have similarities with mouse and human β-defensin-2 (BD-2), contribute to decreased bacterial load (Lange *et al.*, 2018). The oxidative stress reactions generated to clear the excessive bacteria were antagonised

by induced gene expression *gst*, which acts as an antioxidant molecule to maintain cellular homeostasis (Mone *et al.*, 2014; Nathan & Cunningham-Bussel, 2013).

5. 1. 2. The larvae model for the evaluation of virulence

In continuation of the cell-based assay in chapter four, here I used this larvae model to exam the virulence properties of all mutant strains and their corresponding wild types that had been tested in Chapter 4.

In addition, I used this model to investigate the potential role of the *yadA* gene in virulence in the AIEC strains. YadA is a non-fimbrial adhesin originally identified in *Yesinia enterocolitica* (Mikula *et al.*, 2012). YadA belongs to the members of the trimeric autotransporter adhesin family, commonly referred to as mandatory homotrimeric proteins encoded by virulence plasmid of *Yersinia* (Atkinson & Williams, 2016). YadA mediates adherence to several types of cells, including epithelial cells and macrophages, and can act as a haemagglutinin (El Tahir & Skurnik, 2001).

In vitro, YadA exhibits different functions with respect to its position on the surface (China *et al.*, 1993). YadA increases the hydrophobicity of the surface (Martinez, 1989), and facilitates autoagglutination (Mikula *et al.*, 2012; Skurnik *et al.*, 1984). YadA attaches collagen fibres and fibronectin on the epithelial cells and in the extracellular matrix (Emody *et al.*, 1989; Heesemann & Grüter, 1987; Schulze-Koops *et al.*, 1992; Yanisch-Perron *et al.*, 1985). YadA also prevents interferon's anti-invasive function; treatment of tissue culture cells by interferon prevents *Salmonella* and *Shigella* invasion, but not YadA^{+} *Y. pseudotuberculosis* (Bukholm *et al.*, 1990). Eventually, YadA may elicit a humoral immune response, which offers bactericidal activity by promoting membrane attack complex of the complements (Balligand *et al.*, 1985; Pilz *et al.*, 1992).

Bacterial pathogens succeed in counteracting host immunity and modifying cellular pathways, which allows them to benefit from mechanisms whereby effector proteins

are delivered by types III, IV, and VI secretion systems to the host cytosol, and extracellular toxins are delivered as well (Alouf, 2000; Galan, 2009). However, successful infection requires bacteria to attach closely to the host. This is because effector and toxin proteins cannot be secreted without contact. Therefore, adhesion is a major aspect of bacterial colonisation. It facilitates attachment to cell surfaces, hence creating suitable growth conditions, as well as underpinning toxin and virulence factor release (Stones & Krachler, 2015).

Studies on mouse models indicate that *YadA* knockout strains of *Yersinia* lacked virulence and did not produce infection (Di Genaro *et al.*, 2003; Schutz *et al.*, 2010). One way to account for these results is by referencing the fact that a specific type III secretion system (T3SS) exhibits sub-optimal performance in delivering the effector protein known as *Yersinia* outer protein (Yop). This stems from the way the process necessitates adequate attachment to host cells. Resistance to phagocytosis is lost in the absence of adhesion (Cornelis, 2002; Viboud & Bliska, 2005). While a paralogue of *yadA* is present in the AIEC strain LF82 genome accession (NC_011993.1) its role in AIEC virulence has not been studied. Therefore, I have undertaken its investigation in this chapter.

5. 1. 3. A *Galleria mellonella* model to test antipathogen antimicrobial drugs

Galleria mellonella model is an alternative for rapid *in vivo* assessment of antimicrobial drug efficacy and reduces the likelihood of an antimicrobial agent performing well *in vitro* studies from progressing to poor output in a mammalian model. Therefore, the *Galleria mellonella* model will serve as an additional pre-screening experiment to reduce the number of antimicrobial drugs used in mammalian models (Tsai *et al.*, 2016).

Galleria mellonella larvae can be reliably injected with specified bacterial doses resulting in reliable levels of survival/mortality. Therefore, it is easy to determine a dosage that will not kill the larvae instantly but leads to increased mortality over a reasonable course of time, e.g. one to three days. Antimicrobial agents can be used in different treatment regimens including total dosage, number of doses, and schedule of treatment. Many experiments have used single doses of medication, usually given between 30 min and 2 hours after larvae have been infected with the test pathogen. In certain cases, the agent was administered immediately following infection or even before the infection (Betts *et al.*, 2014; Dean *et al.*, 2011; Thomas *et al.*, 2013). Systemically, the antimicrobial agent can be administered by injecting it directly into the hemocoel which closely mimics the conventional route of administration used in mammalian models. Many experiments used antibiotic combinations and demonstrated synergism when applied to the *Galleria mellonella* larvae that were infected. For example, gentamycin and daptomycin injected 1 hour after infection with vancomycin-sensitive *Enterobacter faecalis* or *E. faecium* resistant to vancomycin was considerably more effective than either antibiotic administered alone at the same doses (Luther *et al.*, 2014). Unfortunately, Tsai *et al.* (2016) have found the synergy effects observed between antibiotic combinations *in-vitro* screens did not occur in the *Galleria mellonella* model.

Thus, the *Galleria mellonella* model has provided a useful *in-vivo* application for antibiotic effectiveness screening. For the *Galleria mellonella* larvae infected with *Francisella tularensis,* the significance of proper timing of drug administration has been shown (Tsai *et al.*, 2016).

5. 2. Aims of the experiments

Application of *Galleria mellonella* model to evaluate the virulence property of the aforementioned mutant strains and their corresponding wild types, and assess the potential protection of larvae from AIEC infection by *E. coli* K12 strain that expressed *YadA in trans*. In addition, evaluating geraniol's antimicrobial efficacy against AIEC (HM605) as a novel therapy to combat the antibacterial resistance.

5. 3. Results

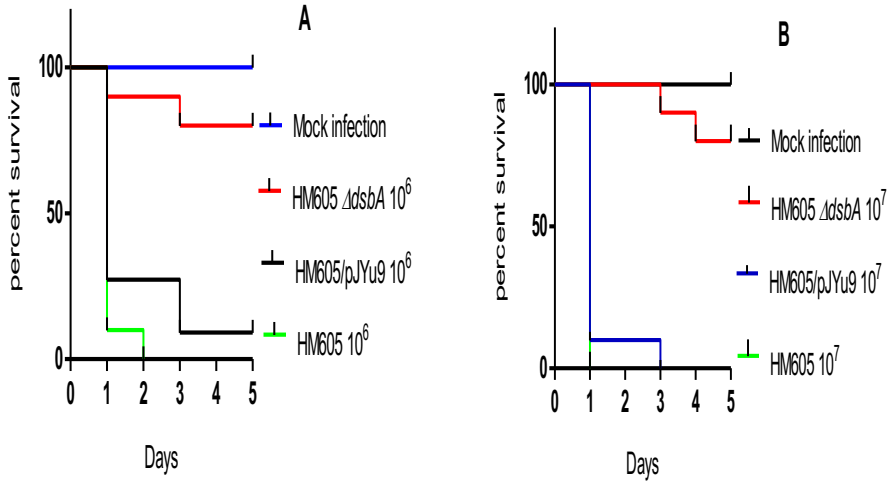
The larvae were 250 mg on average, 15 to 25 mm in width and 2 cm long, cream in colour, without grey marking, and with a minimum of speckling. To find the mortality rates of the wild type AIEC and *ΔdsbA* mutant strains, ten larvae were challenged by 10^6 and 10^7 CFU (Figure 5. 1 A, B and C, D, E, respectively). The larvae were monitored daily for 5 days. Dead larvae were high in melanin pigmentation and did not respond to touching. Three independent experiments were conducted for each dose and the mean was taken for analysis. The Kaplan-Meier survival curve was used for the analysis of data. Images of the larvae were taken for presentations.

5. 3. 1. AIEC (HH427 & HM605) infection

With the higher dose of 10^7 CFU, the wild type HM605 and the complemented strain, *ΔdsbA*/pJYu9, killed all larvae on the first day (Figure 5.1B). With the lower dose of 10^6 CFU, strain HM605 killed all larvae in the second day (Figure 5. 1 A). For wild type HH427, the higher dose of 10^7 CFU killed 70% and 90% of the larvae at the first and third day, respectively, (Figure 5.1E), and the lower dose of 10^6 CFU killed 50% and 90% at the third and fifth day (Figure 5. 1 D), respectively. In contrast, the mock infection, where larvae injected with 10 µl of PBS (mock infection) all stayed alive until day 5. Indicating that there were significant differences between wild type and

dsbA mutant strains (*p*= 0.0057and *p*<0.0001 respectively for low and high bacterial doses).

HM605 *ΔdsbA* mutant strains produced a survival rate of 90% on the first, third, and fourth days when using the lowest and highest dose of 10^6 & 10^7CFU of respectively. By day 5, the survival rates were 80% for both doses (10^6 and 10^7), respectively. There was a significant difference between the *dsbA* mutant wild type and complemented strain (*p*= 0.0007, *p*= 0.0011, and *p*<0.0001 for low and high bacterial doses, respectively (Fig 5. 1 ABC) by using Gehan-Breslow-Wilcoxon Test of Graph Pad Prism 5. The killing rate of HM60 mutant strains for both doses used was similar, but the death of larvae for high dose was one larva each day in the third and fourth day, while the low dose was in the first and third days.



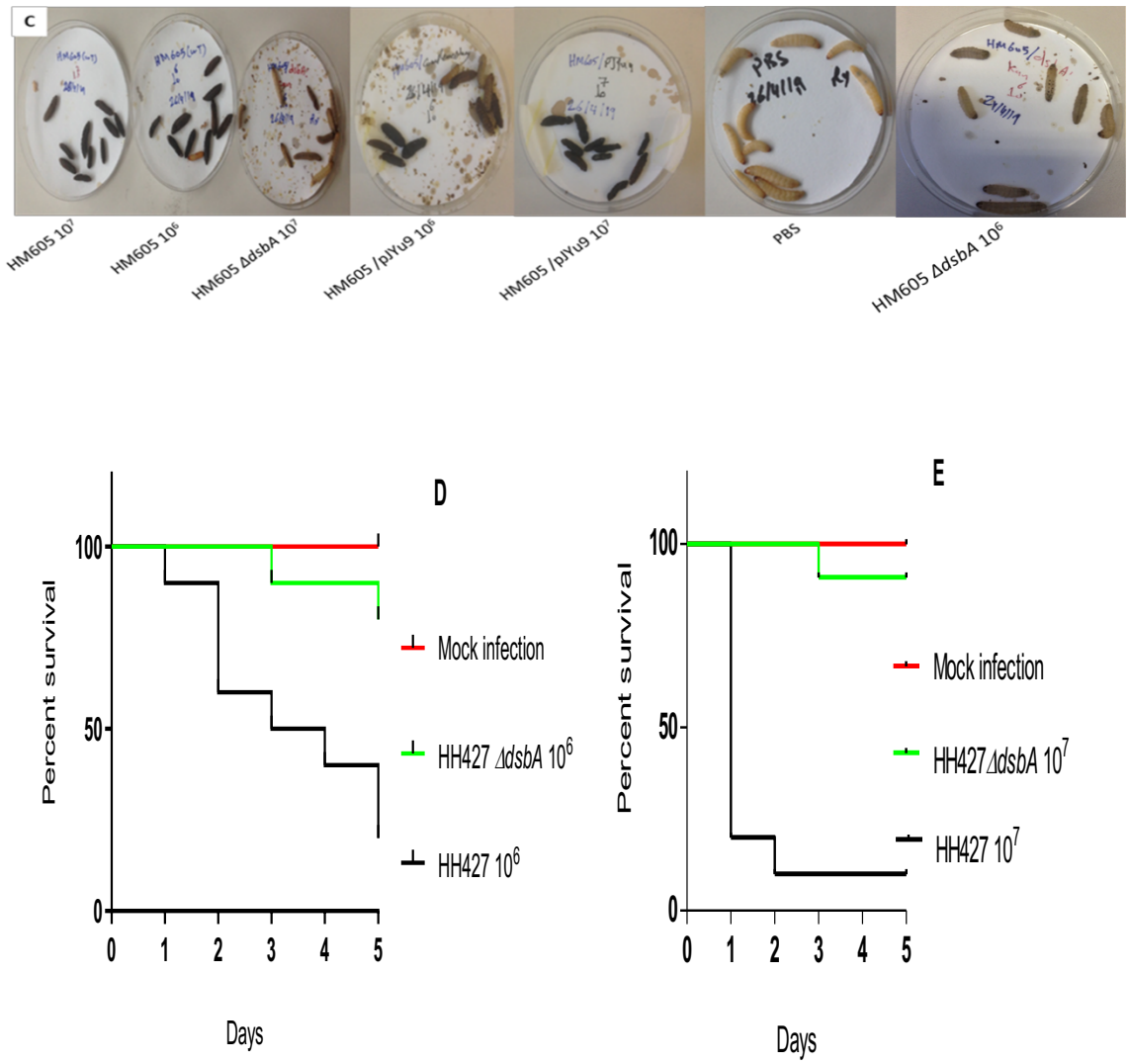
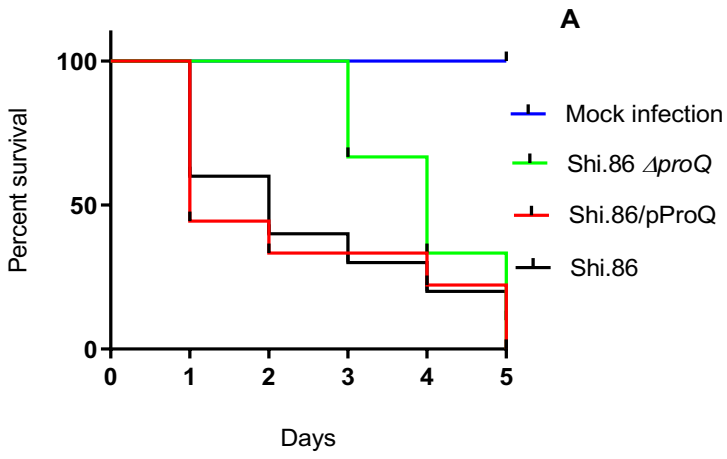


Figure 5. 1. ***In vivo Galleria*** **moth larvae model for assessing virulence of AIEC strains, HH427 and HM605.** Ten larvae had been injected with indicated strains for 10^6 (**A** and **D**) and 10^7 (**D** and **E**) of bacteria per larvae. Larvae survival rates were scored up to five days each day. The results are pooled from at least three independent analyses for the means. p = 0.0007, p = 0.0011 and p = 0.0057 for (**A, D)** and P<0.0001 for **(B, E**) respectively, was found between the WT and complemented strains with the *dsbA* mutants in all cases by using Gehan-Breslow-Wilcoxon Test of Graph Pad Prism 5. **C.** Images of *G. mellonella* Larvae 2 days after injected of 10^7 CFU of indicated strains or mock injection with sterile PBS.

5. 3. 2. *Shigella Galleria* infection

For the *Shigella sonnei* strains (*ΔproQ, ΔicsA,* and *ΔmxiD* mutants and the wild type) larvae were challenged with 10^5 CFU of each strain; 10 larvae per group. The same quantity of saline was used for mock-infection. On the first day, approximately 50% of larvae were killed by wild type, and 70% were killed on the third day with all remaining larvae dying on the fourth day. The complemented strain, *ΔproQ*/*pProQ*, produced a similar killing rate; only the first day had a slightly lower killing rate than the wild type.

The survival rates were much higher when larvae were infected with the *ΔproQ* mutant; on the first day 10% of larvae died and on the following day, 20% died for *proQ* strains (Figure 5. 2 A, B). With *ΔicsA* and *ΔmxiD* strains, 10% were killed on the third day and 20% on the day following (Figure 5. 2 C). 80% of the larvae survived by day 5 (Figure 5. 2 A, B, and C). Statistical differences were found between the wild type and the mutant strains (p =0.0004 for *proQ* and p = 0.0038 for *ΔicsA* and 0.0108 for *ΔmxiD*), while it was found 0.0001 between *ΔproQ* and complemented strains by using Gehan-Breslow-Wilcoxon Test of Graph Pad Prism 5. Also, there were no significant differences between the wild type and complemented strains.



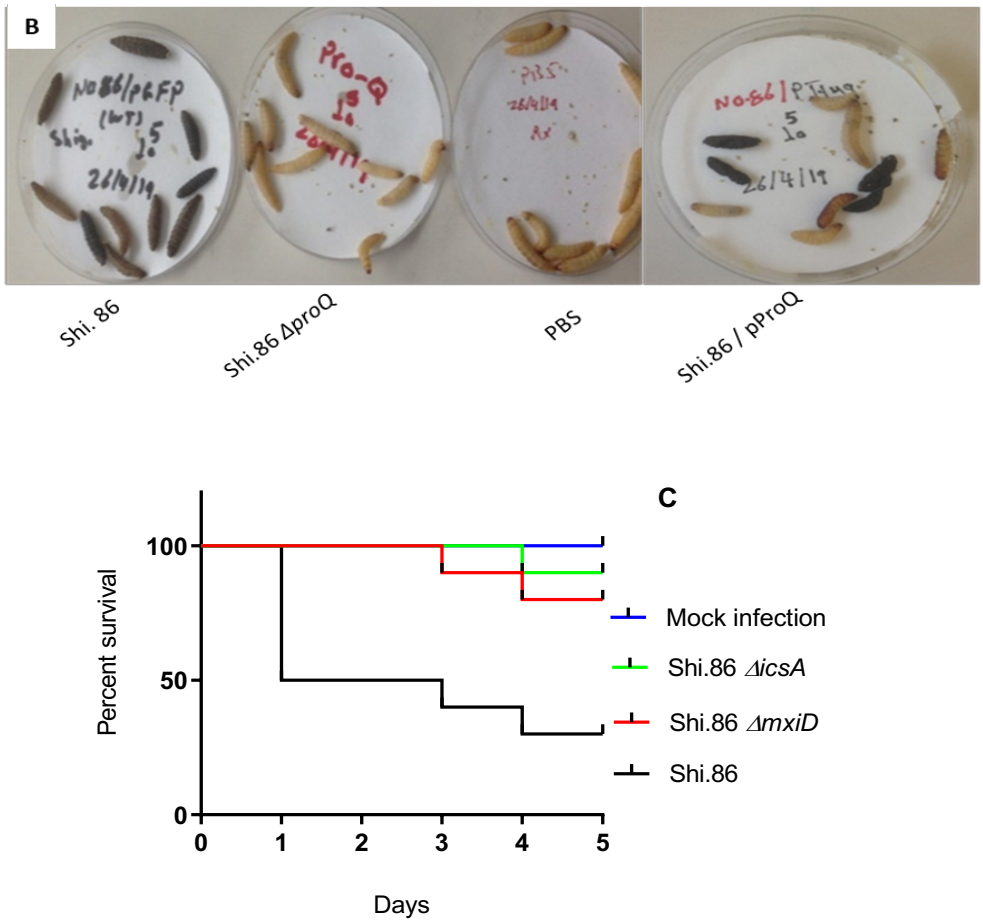


Figure 5. 2. *In vivo Galleria* moth larvae model to evaluate the virulence of *Shigella* strains. Ten larvae were injected with indicated bacteria strains (10^5 bacteria per larvae). Larvae survival rates were scored every day for up to 5 days. Results are pooled for the means from at least three independent analyses. *P*-values were found to be 0.0004, 0.0001 for **A** and (0.0038 for *ΔicsA* and 0.0108 for *ΔmxiD*) for **C** between WT and complemented strains with all mutant strains respectively, by using Gehan-Breslow-Wilcoxon Test of Graph Pad Prism 5.

B. images of *G. mellonella* larvae 2 days after injection of 10^5 CFU of *Shigella* strains or mock injected with PBS.

5. 3. 3. The role of AIEC YadA in virulence

The researcher wanted to generate a deletion mutant to investigate yadA in virulence but repeated attempts were failed using the red lambda system on all three available AIEC strains. Also was tried P1 transduction by transducing the *yadA*-kan marker from a mutant avian pathogenic *E. coli* (APEC) strain, which was generated previously

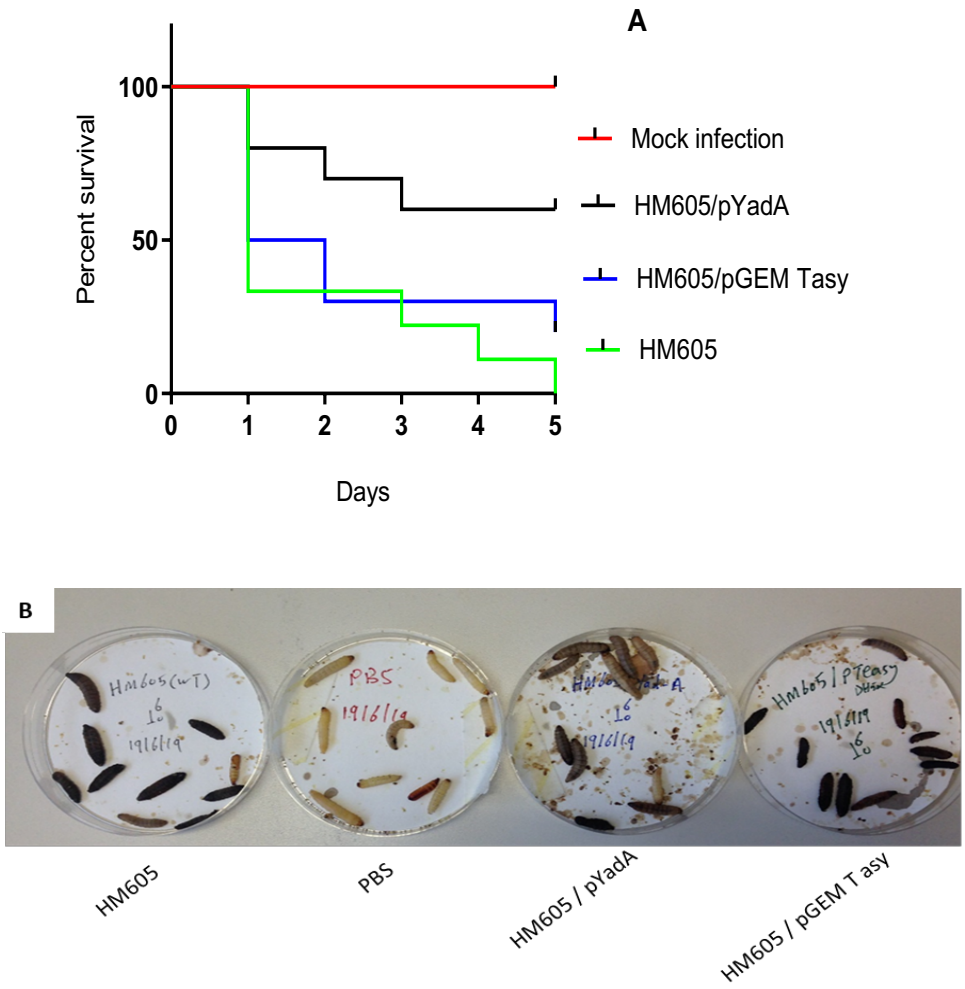


Figure 5. 3 (AB). *In vivo Galleria* moth larvae model for assessing yadA- AIEC clone strain virulence. Ten larvae were injected with indicated bacterial strains (10^6 bacteria per larvae). Larvae survival rates had been scored for up to 5 days each day. Results are pooled from at

least three independent analyses for the means. The variation was found significant among experimental groups *P* = 0.0158 by using the Gehan-Breslow-Wilcoxon Test of Graph Pad Prism 5.

B. An image of *in vivo Galleria* larvae after two days from injection with AIEC strain and an equal dose of PBS.

in Yu's group (unpublished data). Therefore, I set up to clone the *yadA* from LF82 in *E. coli* K12 DH5α and use the resultant strain for competition assay using the *Galleria* larvae.

The 4947 bp coding sequence of *yadA* was cloned to pGEM-T-Easy and transferred to DH5α. Restriction digestion was done to verify the correct orientation, i.e. the *yadA* coding sequence was driven by the *lacZ* promoter. In the competition assay, 5 µL of the DH5α/pYadA was injected into the last left proleg and 5 µl of the wild type HM605 were injected into the last right proleg. Experimental data are shown in Fig. 5. 3 AB. HM605 (10^6) killed 60% percent of larvae in day one and killed 90% by day 5. Co-injection of 10^6 of DH5α/pYadA reduced death rate to 10 and 60% in the first and fifth days, respectively. This was a significant death reduction among strains *P*= 0.0158 using Graph Pad Prism 5's Gehan-Breslow-Wilcoxon Test. In contrast, the co-injection of DH5α/pGEM-T-Easy did not achieve statistical significance although a small reduction death rate was noted.

5. 3. 4. Geraniol protection assay in *Galleria*

Continuing with the cell line and biofilm formation in Chapters 3 and 4, further *Galleria mellonella* larvae model was used to assess geraniol activity against AIEC.

The dose of geraniol at the concentration (0.075M) was mixed with bacteria (10^7) before injection. 10 µL of the mixture was injected into the last proleg, in a different way than (Mirza *et al.*, 2018). Data were presented in Figure 5. 4 AB. Positive control of wild type HM605 was killed all the larvae in the first day, in comparison to co-injected with geraniol at 3.5mg/kg; where it reduced the death percent at 90% for the 3.5mg/kg in the first with killing rate 10%, revealing that there was a significant difference between wild type non treated and treated at the concentration used with $P<0.0001$ by using Gehan-Breslow-Wilcoxon Test of graph pad prism 5. The remainder of the larvae kept alive until the experiments ended (please refer to Figure 5. 4 B).

Injection with geraniol (alone) or PBS concentration as control, larvae were kept alive for five days except that one larva died without statistical differences on the first day for geraniol. This has led to the survival line being mixed with geraniol used in larvae treatment.

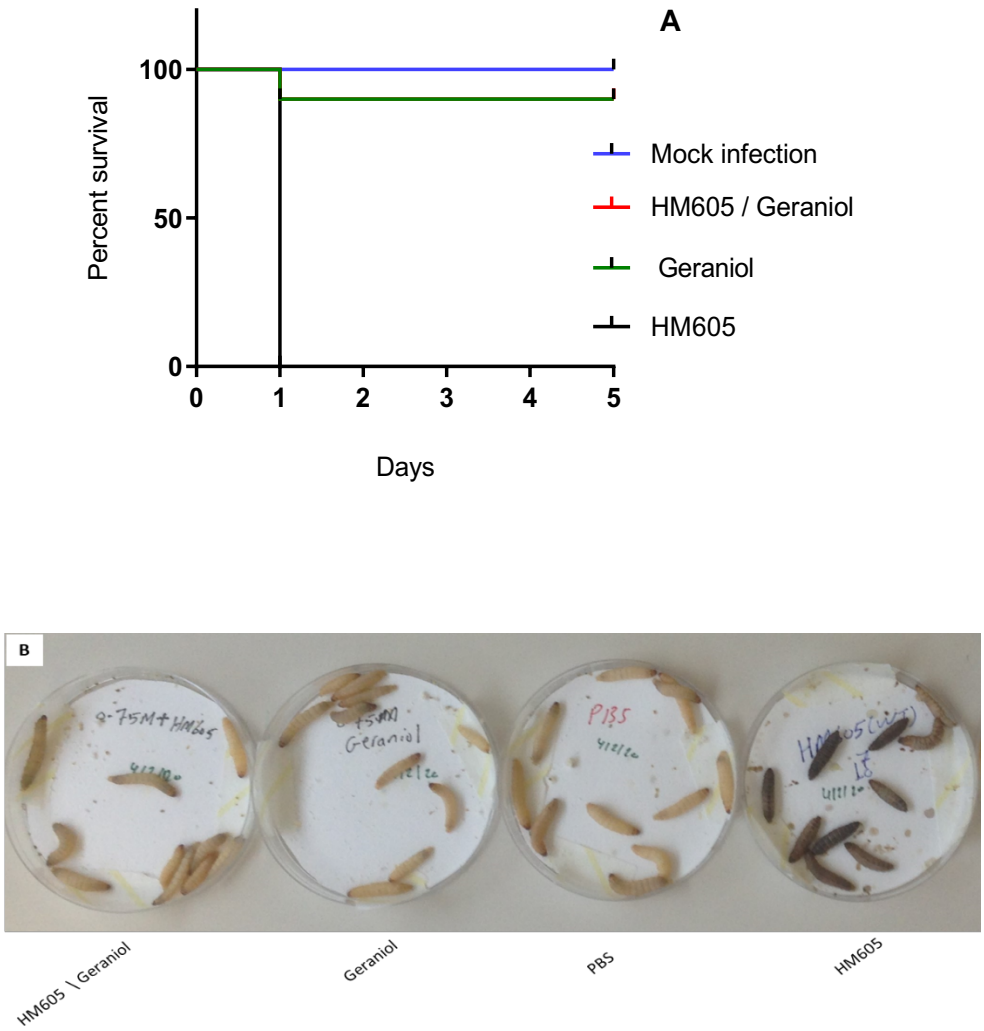


Figure 5. 4 AB. (A) Larvae geraniol safety assay,10 µL of the mixture (HM605-10^7 + geraniol) was injected at the specified doses to the right of each larva's last proleg. For five days, all larvae have been observed. Melanisation and loss of mobility are predictive for larvae death. Experiments were replicated three times (n=3), and the graphs were generated using pooled data. Differences between untreated and treated groups infected with AIEC (HM605) were measured using the Gehan-Breslow-Wilcoxon Test with a significance value set at *P<0.0001*.

(B) Image of *Galleria mellonella* larvae. One-day *Galleria mellonella* larvae were injected simultaneously with a dose of AIEC 10^7CFU — HM605(WT) plus indicative doses of geraniol with an equivalent sterile buffer volume or geraniol alone in the last proleg.

5. 4. Discussion

5. 4.1. *Galleria* larvae model for evaluating and comparing the virulence of AIEC and *Shigella*

Here, the researcher adopted the model of the *Galleria* larvae to evaluate AIEC and *Shigella* virulence. *G. mellonella* larvae are killed by wild type AIEC and *S. sonnei,* in a dose-dependent manner, whereas mutations in *dsbA, proQ, icsA* and *mxiD* of *S. sonnei* and *dsbA* of AIEC significantly increased larval survival (Figure 5. 1 A-E and Figure 5. 2 ABC). Both *S. sonnei* and AIEC are Gram-negative pathogenic lineages of *Escherichia coli* (Barnoy *et al.*, 2017). As both AIEC and *S. sonnei* are invasive to macrophages as reported previously (Glasser *et al.*, 2001; Kuwae *et al.*, 2001; Migliore *et al.*, 2018) and I have demonstrated again in chapter four, presumably invasion and proliferation within the larvae haemocytes are one of the important mechanisms in larvae killing. As to *S. sonnei*, a previous study showed that killing of *G. mellonella* larval was strongly correlated with the bacteria's ability to invade haemocytes and to trigger apoptosis of the haemocytes (Mahmoud *et al.*, 2016). A reduced invasion of haemocytes and apoptosis (as in macrophage infection) as a result of the deletion of the genes can be envisaged. Apoptosis induction in hemocytes is comparable with apoptosis induction in human macrophages because of the activation of caspase I by secreted IpaB (Chen *et al.*, 1996; Mahmoud *et al.*, 2016).

According to (Harding *et al.*, 2012), vertebrate macrophages have similar characteristics to haemocytes. Homologues to human caspases are found in *G. mellonella* larvae, which involves tissue remodelling during metamorphosis (Khoa *et al.*, 2012). More recently, a cDNA encoding a caspase has been cloned and sequenced from the larvae midgut (Barnoy *et al.*, 2017). This larvae caspase share similarity with human caspase 3, which is the main component initiating apoptosis (Porter & Janicke, 1999). The *G. mellonella* is also found to have homologous of the human NADPH

oxidase (Khoa *et al.*, 2012; Renwick *et al.*, 2006). Therefore, mechanisms used by mammalian neutrophils and macrophages, including oxidative bursting, lysozyme production, and numerous antimicrobial peptides that kill ingested microbes, are also in play in *Galleria* haemocytes (Barnoy *et al.*, 2017).

In contrast to *S. sonnei*, AIEC is persistent in macrophage and dendritic cells owing to S-nitrosylation in inducing caspase 3 degradation that leads to accumulation of bacterial load in the cells (Dunne *et al.*, 2013). However, for replication of AIEC LF82 bacteria inside J774 macrophages, the acidic vacuolar environment is essential (Bringer *et al.*, 2006a). Based on the similarity between macrophages and haemocytes the possible mechanism for the AIEC to proliferate and accumulate inside the larvae haemocytes may occur. One can imagine that eventually the haemocytes will be killed due to the heavy intracellular bacterial load. This type of cell-killing would be slower than apoptosis. This might explain why a high dose of AIEC, in comparison with *S. sonnei*, is required to kill larvae. It has been reported previously (Bringer *et al.*, 2007) and demonstrated in Chapter 4 of this study that the deletion of *dsbA* resulted in the impaired intracellular proliferation of AIEC strains. The deficiency in the proliferation of the AIEC bacteria inside haemocyte is likely the explanation as to why the *dsbA* mutant is significantly attenuated in this larvae model.

AIEC does not have T3SS but possesses T6SS, which has been studied for its role in virulence (Ma *et al.*, 2009) or host immunomodulation (Chow & Mazmanian, 2010). Recent findings have related T6SS to inter-bacterial interactions ranging from self vs non-self-discrimination (Alteri *et al.*, 2013; Wenren *et al.*, 2013), competitive growth in mixed-culture biofilms (Schwarz *et al.*, 2010), to bactericidal activity (Hood *et al.*, 2010; MacIntyre *et al.*, 2010). T6SS is able to translocate proteins into both eukaryotic and prokaryotic cells. Qin and his groups showed that an atypical *Dsb* of *Francisella tulariensis*, FipB (*FtDsbA*), which shows oxidase, isomerase, and chaperone activity, affects the cycle of virulence by affecting the proper assembly of the type 6 transport system (Qin *et al.*, 2016). Moreover, for many pathogenic bacteria, the ability to bind

to a wide range of surfaces is important. The process involves specific cell organelles, such as adhesion pili on the cell surface. The proper assembly of *E. coli* pili depends solely on the generation of disulfide in pilus or chaperones (Crespo *et al.*, 2017). As a result, In reviewing previous publications, and from the above and in a similar manner, I supposed that the possible mechanisms for attenuation of virulence are as following: deletion *dsbA* will lead to inactivation type 6 secretion system and inhibition of the adhesion. Thus, further study would be recommended to investigate the importance of T6SS in AIEC.

The importance of the genes under investigation has been demonstrated again using this moth larvae model for their necessity in the virulence. The results in the larvae model were consistent with the findings in biofilm formation and cell-based assays in chapter 4, and an *ex vivo* model in following chapter 7.

5. 4. 2. Competition assay indicated that *YadA* was important in AIEC pathogenesis

YadA was originally identified in *Yersinia* (Achtman *et al.*, 1999; Forman *et al.*, 2008). Yu's lab generated a *yadA* mutant AIEC strain, which was attenuated in virulence (unpublished data). Although mutating *yadA* gene was not achieved, the researcher was able to be obtained data to implicate the role of the *yadA* homolog from AIEC in virulence presumably through adherence. The fact the co-injecting DH5α/pYadA with the wild type AIEC strain significantly increased larval survival rate indicates that DH5α/pYadA is competing with the wild type AIEC, very likely by reducing the latter in adherence of haemocytes and other host cells or reducing wild-type bacterial numbers similar to *Salmonella* and *Shigella* by preventing anti-invasive interferon function (Bukholm *et al.*, 1990).

5. 4. 3. *mxiD, icsA, and Shigella proQ*, larval infection

The *Shigella* T3SS is a needle-like injection machinery encoded by about 25 genes located on a large 230 kb plasmid in the *mxi, spa* and *ipa* operons (Buchrieser *et al.*, 2000). The proteins secreted via the T3SS after assembly of the external needle fall into two main categories: translocators and effectors (Collmer *et al.*, 2002; Cornelis, 2002). Translocators assemble into the host cell membrane after host cell contact, forming a pore complex or translocon that triggers the subsequent export of effectors (Mota *et al.*, 2005).

The first step in the T3SS assembly is concerned with the creation of the basal, seven-ringed body, which covers the bacteria's inner membrane, periplasm, peptidoglycan surface, and outer membrane. The main component of the basal body's outer membrane rings is the secretin protein, MxiD, which is transferred through the secretion pathway to the periplasm (Blocker *et al.*, 2001; Kuwae *et al.*, 2001). The formation of the outer membrane rings requires the penetration of the outer membrane-based peptidoglycan meshwork. Periplasmic and inner membrane rings consist of the lipoprotein MxiJ and the membrane protein MxiG. These proteins are anchored or inserted into the inner membrane, and they interact via periplasmic domains with MxiD and MxiM (Sani *et al.*, 2007; Schuch & Maurelli, 2001). From the above, the present work concluded that the *mxiD* gene is critical for the activity of T3SSs, and so the deletion *of the mxiD* gene will lead to inactivation of the T3SS, which prevents the larvae from dying. Due to the key role of MxiD in TTSS, it is likely that the deletion of *mxiD* would greatly reduce the invasiveness of the larvae haemocytes. Therefore, significantly increased larval survival is expected.

Shigella IcsA is multi-functional. 1, IcsA mediates TTSS-dependent attachment (Brotcke Zumsteg *et al.*, 2014), 2, IcsA enables intra- and inter-cellular motility by polymerase host cell actin (Goldberg & Theriot, 1995; Mahmoud *et al.*, 2016) and 3,

triggers autophagy via its autophagous recognition domain (Ogawa *et al.*, 2005). While there lacks information as to whether the larvae haemocytes also use autophagy as a defence mechanism, reduced cell attachment and actin-based motility can be envisaged to the causes that the *icsA* deletion mutant is significantly attenuated in thus larvae model.

The lack of *proQ*-mediated gene regulation in infectious bacteria triggered metabolic system upregulation in the host, whereas immune, calcium, and G-protein signalling host pathways are downregulated. In this context, mitogen-activated protein kinase (MAPK) signalling is the most strongly repressed host pathway (Westermann *et al.*, 2019). These reactions will lead to the survival of the larvae infected with *Shigella ΔproQ* deletion, and they will kill the larvae that infected with wild type in the same background.

5. 4. 4. Geraniol-*Galleria* protection assay

Galeria mellonella larvae have been used in a number of studies to evaluate the effectiveness of antimicrobial therapies against Gram-negative pathogens (Hill *et al.*, 2014; Peleg *et al.*, 2009; Seed & Dennis, 2008). The efficacy of different antibiotic regimens against larvae infected with *Acinetobacter baumannii*, for example, accurately reflected the established resistance trends of the strains used (Peleg *et al.*, 2009). Desbois and Coote have confirmed that antibiotics are also successful in the larva model for the treatment of Gram-positive bacterial infections (Desbois & Coote, 2011).

Nevertheless, no published studies have tested the suitability of *Galleria mellonella* larvae to test AIEC virulence and the effectiveness of antimicrobial therapies against AIEC.

Its infection characteristics were described first to determine whether the *Galleria mellonella* larvae were a suitable model for the study of AIEC pathogenesis. Killing substantially relied on the number of AIEC cells injected into larvae. Results indicated a positive correlation between the number of AIEC cells inoculum and larval death rates. These data suggest that AIEC pathogenesis requires infection with live bacterial cells in larvae of the *Galleria mellonella*. Similar results have been previously described for other pathogens (Desbois & Coote, 2011; Mahmoud *et al.*, 2016).

In our research, AIEC strains with profiles of antimicrobial resistance were used to distinguish the effect of geraniol treatment as antimicrobials on AIEC strain-infected larvae. Our study showed that the antimicrobial efficiencies on infected larvae are closely correlated with the known *in vitro* strain susceptibility of the drug. Geraniol treatment of a lethal AIEC infection greatly increased *Galleria mellonella's* survival rate. Such results suggest that the *Galleria mellonella* model will prove useful in determining the *in vivo* efficacy of new antimicrobials.

The injected dose was selected on the basis of the previous geraniol screening test performed by (Mirza *et al*., 2018) whereas the injection method was selected differently; where geraniol was found to be safe for larvae at present concentrations (0.75 M). This was confirmed by injecting this dose to the larvae alone where no death rate was found.

A single dose of geraniol (3.5mg\kg) and (10^7) of HM605- bacteria were injected simultaneously together into the last pro leg of the larvae and monitored for five days. Indicating that there were substantial differences between larvae that infected untreated compared to those treated with geraniol and that clarified geraniol's anti-microbial activity in larvae protection.

Owing to the similarities of haemocytes with macrophages and neutrophils, geraniol may exert similar activity against AIEC bacteria inside the larvae haemocytes. Where HtrA/DegP protein has been found to be dependent on DsbA for its protease function (Skorko-Glonek *et al.*, 2008). In addition, HtrA/DegP has been shown to be important for the phagolysosomal survival of AIEC (Bringer *et al.*, 2005). Consequently, binding

geraniol to the active site of DsbA in the phagolysosomes would, in turn, deprive the internal disulphide bond of HtrA, resulting in the mutant's inability to cope with acidic environmental stress.

In summary, the *Galleria* larvae model was used to assess the virulence of AIEC and *Shigella*. Notably, the results from the larvae model are consistent with those of the biofilm formation and cell-based assays presented in Chapter 4, as well as with the *ex vivo* model given in Chapter 6.

One of the most noteworthy findings reported in this chapter is that, with regard to the manner in which the removal of *dsbA* resulted in impaired intracellular proliferation of AIEC strains (as reported in Chapter 4 of this study), this may be attributable to the deficiency in the proliferation of AIEC bacteria within haemocytes. The results of the competition assay undertaken in this chapter revealed that yadA performed a critical function in AIEC pathogenesis, while it was also concluded that the *icsA* and *mxiD* genes are critical for the activity of T3SSs. Hence, a significant increase in larval survival is anticipated with the deletion of both genes. Significantly, the situation with mutated or inactivated strains is not the same.

Finally, the feasibility of a geraniol-*Galleria* protection assay was evaluated. At the outset, the suitability of *G. mellonella* larvae for assessing AIEC virulence and the effectiveness of antimicrobial therapies against AIEC was explored, and the results indicated a positive correlation between the number of AIEC cells inoculum and larval death rates. Noteworthily, the results indicated that the antimicrobial efficiencies on infected larvae are closely correlated with the known *in vitro* strain susceptibility of the drug. Altogether, the results are promising because they indicate that the proposed *G. mellonella* model, which has not previously been used to assess AIEC virulence and the effectiveness of antimicrobial therapies against AIEC, is both feasible and potentially valuable, particularly in terms of assessing the *in vivo* efficacy of novel antimicrobials.

The purpose of the next chapter is to build on the present results by discussing the results from an *ex vivo* evaluation of virulence and potential drug interventions using mouse intestinal organoids.

Chapter Six. *Ex vivo* evaluation of virulence and potential drug intervention using mouse intestinal organoids

6. 1. Introduction

6. 1. 1. Intestinal crypt isolation and growing of organoids

The main features of organoids are that they organise autonomously (e.g. villus and crypt domains), and they consist of cells identical to those present in actual organs (e.g. enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and stem cells). Cells contained within the organoids function the same as *in vivo*, for example, Paneth cells can transmit niche signals to stem cells and produce antimicrobials upon stimulation (Lancaster & Knoblich, 2014). Due to these features, the cells can be structured three-dimensionally *ex vivo* in a similar way as they are in the organ *in vivo*. The developed structure comprises the various types of cells occurring in the organ in question, and the cells can partly undertake the functions they usually perform in that organ. As exemplified by Sato *et al.* (2009), the mouse intestinal organoid is a standard organoid that develops as an epithelium with one layer structured into domains that mimic the *in vivo* structure of the intestinal crypt-villus, encircling a cystic lumen and displaying the different types of intestinal cells, including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and stem cells.

The culturing of small intestine crypts in Matrigel prompted their closure and development into organoids (Stelzner *et al.*, 2012). These initial organoids were transparent, circular structures that began budding within two to three days, leading to the formation of enteroids after seven days, with multiple structures resembling crypts (Stelzner *et al.*, 2012). It was observed that culturing of the isolated crypts in a medium without R-spondin-1 (a Wnt analog) caused them to cease development and

die following one day of culture, despite having initially closed to give rise to organoids. Significant morphological alterations, with epithelial destabilisation and dysfunctional lumen development, made it possible to differentiate between living and dying organoids, the latter being labelled as 'disrupted organoids'. In addition to organoid development, R-sponding-1 was also found to be essential for enteroid survival, since identical modifications in morphology were observed when R-spondin-1 was removed from mature enteroids (Stelzner *et al.*, 2012).

Stem cell (SC) -derived organoids (LGR5 was co-expressed with homeobox gene 'CDX2' in gut organoids) are responsible for conveying considerable levels of proteins that undergo native expression in the gut. Moreover, they perform critical functions in the context of drug transportation and metabolism. In light of this, when examining the way in which the gastrointestinal tract (GI) absorbs orally administered pharmaceuticals, SC-derived organoids are especially pertinent to consider. It is important to recognise that organoids have the capability to serve as robust preclinical models for examining the efficacy of novel drugs, especially regarding their rapidity, certainty, and cost-effectiveness when compared to live animal research. Furthermore, given that organoids are produced from individuals with varying genotypes, environmental risk factors, and levels of sensitivity to drugs, a pertinent screening mechanism is offered for patient-centred therapies (whether the patient is a human or an animal) (Mochel *et al.*, 2017).

mouse organoids were used for several reasons. Firstly, replication of pathological effects could be distinguished in both mouse models and human cell lines (Sato *et al.*, 2011a; Sato *et al.*, 2011b). Secondly, they enabled the investigation of the interaction between bacteria, including enteric pathogens, commensal bacteria, and probiotics, and the epithelium under closely regulated experimental settings. Thirdly, they enabled the examination of bacterial infection and stem cells *in vitro*. And finally, they allowed the analysis of the ISC response to the molecular mechanisms underpinning bacterial effects, based on *in vitro* wild type and genetically created organoids. This

was identified as a way to create future opportunities to develop human intestinal organoids for the exploration of the interplay between host and bacteria.

6. 1. 2. MTT assessment of intestinal organoids with geraniol

Counting live and/or dead organoids required labour-intensive visual examination via a brightfield microscope, and involved common issues associated with the variability of non-automated protocols. According to Sandercock *et al.* (2015) and Booij *et al.* (2016), although cell death is frequently quantified in established cell culture protocols via MTT staining, this only facilitates an assessment of cell viability and does not evaluate other alterations to the organoids. According to Aslantürk (2018) and Riss *et al.* (2004), the MTT assay evaluates mitochondrial viability and function by identifying succinate dehydrogenase (SDH) activity via the conversion of MTT to a blue crystalline compound that is soluble in DMSO. A spectrophotometer is then used to measure the absorbance of the blue solution, with the optical density (OD) providing a measure of the extent of crystal formation. The SDH molecule is part of the citric acid cycle, transforming succinate and flavin adenine dinucleotide (FAD) into fumarate and FADH2 on the inner mitochondrial membrane. This depends upon a functioning electron transport system, hence, SDH activity is an index of mitochondrial and cell viability.

The tetrazolium salt MTT (thiazolyl blue) is soluble in water, giving a yellow solution in the absence of phenol red. Dehydrogenase enzymes convert the dissolved MTT into water-insoluble formazan (purple) by cleaving the tetrazolium ring. The formazan can be dissolved in isopropanol or another solvent for spectrophotometric evaluation of absorbance as a function of converted dye concentration. In the present work, using murine intestinal organoids as a new model, the MTT assay was used to assess the viability of the cells treated with different concentrations of geraniol and DMSO.

6. 1. 3. Intestinal organoid infection

Intestinal organoids have great potential to afford better insight into the interaction between host and microbes, infectious diseases, and inflammatory response. Moreover, organoids have been employed to model pathogenic infections, interactions between the gut and microbiota, and IBD. Further research should focus on using a greater variety of microbes and should advocate organoids as the common approach for studying interactions between host and microbes. Additionally, the benefits of organoids should be more extensively exploited to investigate microbial reactions in each individual patient (Bartfeld *et al.*, 2015).

Evidence suggests that HIOs have good potential to support an investigation into the interplay between *S. enterica* serovar *typhimurium* and the human intestinal epithelium. Microinjection of the HIO lumen with *Salmonella* permitted the pathogen to penetrate the epithelial barrier and establish itself in vacuoles, whilst also altering the host's transcriptional profile, including the patterns in which cytokines were expressed following exposure to pathogens.

In humans, *S. typhimurium* triggers self-limiting gastroenteritis, while mice are vulnerable to it, it causes systemic disease. Invasion and replication by this pathogen within host cells, including macrophages and epithelial cells, are achieved based on the virulence factors that are encoded in the *Salmonella* pathogenicity islands SPI-1 and SPI-2 (Figueira & Holden, 2012). Several studies reported that host cell genes, including major pro-inflammatory cytokines (e.g. IL-1β, TNF, and IL-8), were upregulated when *S. typhimurium* was injected into intestinal organoids from mouse adult stem cells (ASCs) and intestinal organoids from human iPSC (Forbester *et al.*, 2015; Zhang *et al.*, 2014). Forbester *et al.* (2015) observed that organoids from induced pluripotent stem cells (iPSC) contained intracellular bacteria, with the bacterial invasion protein A (invA) playing a central role in cellular invasion.

The pathogenesis of this disease is associated with genetic susceptibility and constant activation of the mucosal immune system by luminal bacteria and related products. Considered to be pathobionts, AIECs usually occur as symbionts but are capable of becoming pathogenic (Schippa *et al.*, 2012). Moreover, they are unrelated to the intestinal mucosa and are more prevalent in individuals with CD than those without. The AIEC phenotype depends on the intestinal epithelial cells being effectively adhered to and invaded, as well as the ability of the cells to survive and replicate within macrophages (Darfeuille-Michaud, 2002). In the present work, infection of organoids was achieved in two ways: direct and co-culture infection.

As of 2018, researchers have not yet employed organoid cultures for the purpose of modelling IBD in a direct way, however, studies have mobilised organoid cultures in order to examine apoptosis, the impacts of inflammatory cytokines, and mucosal integrity (Grabinger *et al.*, 2014; Leslie *et al.*, 2015; Schwank *et al.*, 2013).

6. 1. 4. Treatment of organoids with different agents

Clarke (2009) described the extensive use of animal models to demonstrate the safety and effectiveness of novel drugs during the preclinical phase of drug development. Drug safety and effectiveness per-oral administration can be affected by numerous factors including bioavailability, the role of gut microbiota, the efflux system, and the first-pass effect (Kang *et al.*, 2013; Li & Nikaido, 2009; Pond & Tozer, 1984). While a wide variety of physiological responses can be observed in animal tests compared to tests on human subjects, Hintze *et al.* (2014) argue that these tests have minimal throughput, are morally questionable and are costly in terms of time, money, manpower and/or quantity of the drug. Furthermore, Rongvaux *et al.* (2014) argue that species differences with regard to animal testing present a significant challenge to the prediction of pharmacodynamic (PD) and pharmacokinetic (PK) responses in

humans. Hence, the yet-to-be achieved the development of a novel *ex vivo* human intestine model is essential in order to represent the 3-dimensional (3D) structure, physical and biological functions, and pathological responses of the human intestine under disease-like stimuli.

Important information relating to the applicability of potential chemotherapeutic drugs may be elucidated by obtaining answers to questions such as: 'does the quantity of crypt-like protrusions stay constant during treatment?', 'are the organoids larger or smaller?', and 'does the cell size vary?'. Clearly, the structural intricacy of organoids presents a challenging aspect previously unseen when using 2D cell cultures.

For each commercially available medication that exists in the present day, at least 250 are not successful in making it past the animal-based preclinical testing phase. In addition, even among drugs that are advanced to the clinical testing phase, adverse secondary effects, often paired with treatment ineffectiveness, contribute to a high rate of failure. Based on this, it is evident that the process of laboratory testing that predominates in the contemporary pharmaceutical industry must be altered in such a way as to safeguard against the needless entrance of unviable drugs into the expensive, ethically difficult, and generally complicated preclinical animal testing phase.

In 2015, *ex vivo* cultures of organoids derived from patient tissues were first subjected to drug screening tests to aid treatment in a case of cystic fibrosis (CF) (Dekkers *et al.*, 2016). This was a watershed moment during which intestinal organoid cultures became a more common approach for disease investigation (Dekkers *et al.*, 2016; Dekkers *et al.*, 2013).

According to VanDussen *et al.* (2014), irregular Paneth cell phenotypes are linked to certain susceptibility alleles in the context of CD. Furthermore, Farin *et al.* (2014) discovered that when interferon-gamma (IFN-γ) was administered to cultures of mouse organoids from the small intestine, the degranulation of Paneth cells took

place, which was linked to programmed cell death. In the context of an intestine in full health, the rapid replacement of Paneth cells is a critical activity. Nevertheless, when IFN-γ is continuously present, the gradual loss of Paneth cells takes place, and the literature suggests that this could be one of the aetiological underpinnings of IBD. Nevertheless, in order to confirm whether the degranulation of Paneth cells is implicated in the IBD pathogenesis, additional research particularly focused on epithelial organoids from IBD patients is required. As demonstrated by Rodansky *et al.* (2015), one of the complications of IBD is intestinal fibrosis, which highlights the importance of the fact that Pluripotent stem cell (PSC)-derived organoids have been applied for the modelling of a fibrosis-like response to transforming growth factor-beta (Tgfb) stimulation, which may be reversible with the administration of anti-fibrotic agents.

Research conducted by Jarry *et al.* (2017) demonstrated that IFN-α was responsible for inducing programmed cell death and, at the same time, indirectly disrupted the intestinal epithelial barrier (IEB) via the activation of the inflammasome. Furthermore, a Th1 response was induced by IFN-α, characterised by elevated numbers of T box expressed in T cells (T-bet positive cells) in the lamina propria, paired with higher levels of IFN-γ. During the study, researchers employed various inflammasome pathway inhibitors in the explant cultures to identify a series of events that disrupted the intestinal IEB.

When conducting research similar to that of Jarry *et al.* (2017), the use of animal models can lead to extreme complexities, for example, certain variables (i.e. drug delivery routes, the interference of drug systemic effects, complications related to the synchronisation of cellular responses in tissues, and the matter of drug clearance and half-life) can prove to be difficult when attempting to identify cause-and-effect relationships with clarity. Nevertheless, studies have demonstrated the following sequence of events: firstly, IFN-α is responsible for activating caspase-1 in epithelial and mononuclear cells; secondly, the epithelial and mononuclear cells give rise to IL18, and subsequently induce IFN-γ secretion by T-bet^{+} T lymphocytes in the lamina

propria; and finally, the previous event gives rise to programmed cell death for the epithelial cell, thereby disrupting the IEB (Osaki & Mills, 2017).

(Jarry *et al.*, 2017) used an *ex vivo* system in the form of a three-dimensional model of a human normal colonic mucosa explant culture, to preserve the tissue structure and at the same time incorporate different mucosal cells (whose interaction can activate innate immunity). This model provided insight into the series of cellular and molecular processes induced by IFN-α. It was observed that homeostasis in the human normal colonic mucosa was disrupted by IFN-α, which quickly caused whole crypts to die. However, this effect was not uniform across tissue samples from various patients, and in 21% of cases, apoptosis was not triggered. Hence, the model effectively replicated the variability in the effects of IFN-α-based treatment on cancer patients, with intestinal conditions as a secondary effect(Jarry *et al.*, 2017).

In research, the production of cytokines and their interaction with immune cells has been prioritised over the direct impact of cytokines on intestinal epithelial cells. The absence of *in vitro* models that provide insight into the different cellular constituents may explain the limited knowledge regarding the direct production of epithelial cells and how they react to cytokines. A recent study was undertaken to improve on this knowledge by employing a three-dimensional model of an organoid culture consisting only of epithelial cells. Small intestinal organoids of murine origin were used to demonstrate that Paneth cells underwent degranulation and died as a direct result of IFN-γ, whereas the other cytokines under investigation, such as IL22, had no effect on those cells (Farin *et al.*, 2014).

This work describes the development and characterisation of an organoid infection and macrophage-enteroid co-culture model consisting of murine enteroid monolayers and murine monocyte-derived macrophages. The work also describes the effective application of the model in the investigation of intestinal epithelial and macrophage interactions and their responses to enteric pathogens, using geraniol as an antibacterial.

6. 2. Aims of the study

The aims of the study were to:

1. Optimise mouse intestinal crypt isolation and culture to organoids.
2. Evaluate organoids as a direct infection *Shigella* model, and AIEC as direct infection or co-culture with RAW macrophage cells.
3. Study the impact of geraniol on AIEC replication inside the macrophage in the co-culture model.
4. Study the effect of using a variety of agents on the organoid.

6. 3. Results

6. 3. 1. Isolation of intestinal crypts and culturing organoids

Small intestinal epithelial cells were collected from male BALB/C mice by caesarean section at 6 to 7 weeks of age and were placed in an ice-cold PBS buffer that did not contain Ca^{2+} or Mg^{2+}, and the mesentery was discarded. The distal half of the murine small intestine was opened longitudinally and flushed out with ice-cold PBS to eliminate the luminal material. Using sharp scissors, the intestines were cut into 1 mm long fragments. The clean pieces of the small intestine were immersed in a chelating agent buffer for 23 to 30 mins to release the crypts, with help from shaking buffer.

Organoids were successfully developed from 50 crypts for every 50 µl Matrigel droplet (Figure 6. 1). At 24 hrs after culturing, the organoids displayed an oval shape (Figure 6. 1 A), and at 48 hrs they displayed an elongated shape (Figure 6. 1 B), while at 72 hours they began to develop buds (Figure 6. 1 C) that constituted the crypt structures. Not all organoids had the same number of buds (Figure 6. 1 B, D, E, F) and some lacked buds, indicating a paucity of uniformity with regard to organoid

development. This issue can be addressed through additional optimisation. On day six, shed epithelial cells were found to aggregate in the lumen of the organoids, most probably due to epithelial turnover, which lasts for approximately six days (Mahe *et al.*, 2013; Sato & Clevers, 2013).

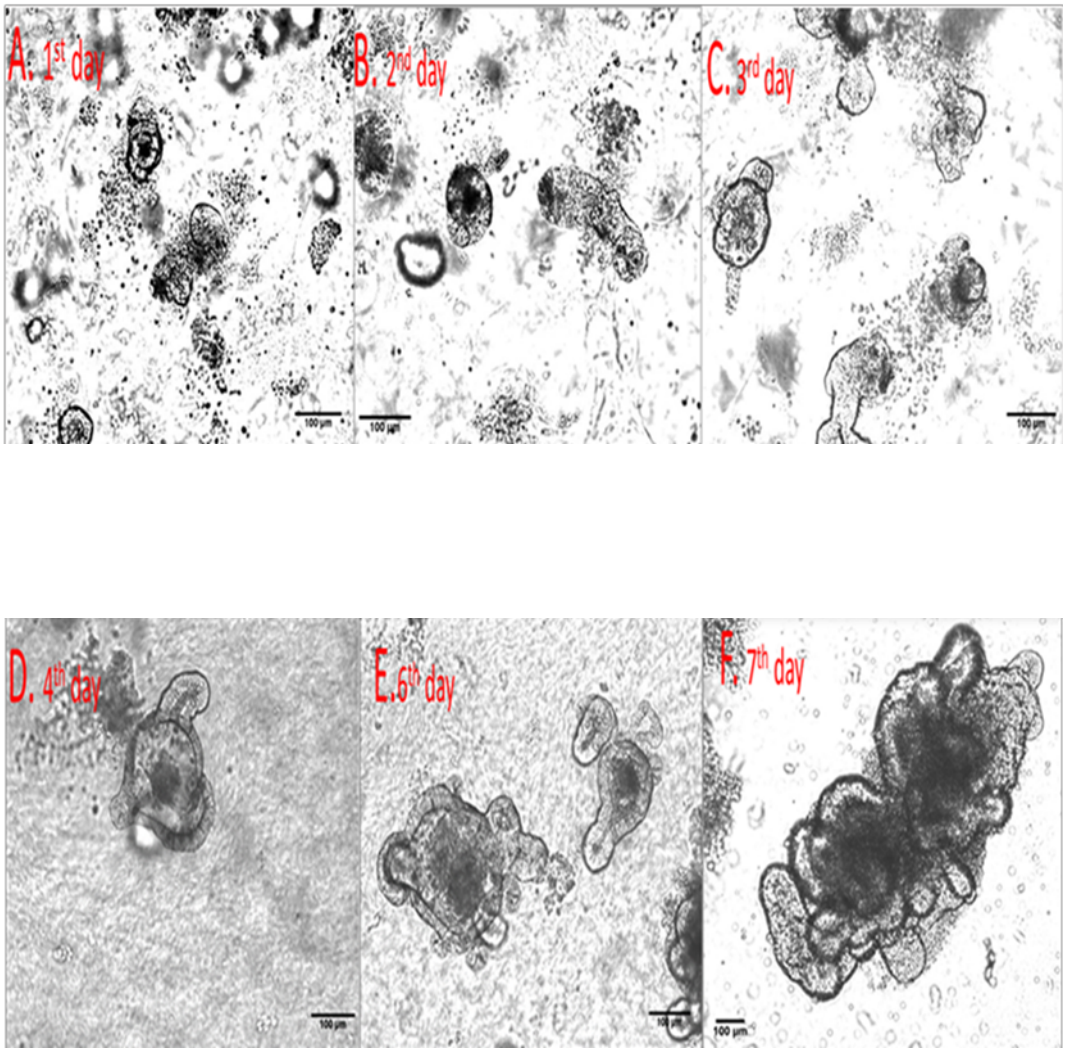


Figure 6.1. Development of intestinal organoids. The mouse intestinal crypts were isolated, and organoids were developed in the Matrigel with supplements under conditions of 37°C and 5% carbon dioxide. Images were taken, ranging from one to seven days (A to F). Inverted microscope (scale bar = 100μm), under a bright-field microscope.

6. 3. 2. MTT analysis of geraniol in intestinal organoids

In pursuit of an objective and effective quantitative methodology for evaluating organoid live/death, the intestinal organoids were stained with MTT, which facilitated the detection of the viable organoids that were capable of converting MTT into a purple formazan precipitate. Subsequently, this allowed for a distinction between dead and viable organoids. Intestinal crypts were isolated, counted and seeded. Before measuring the MTT reduction at 562 nm by using Spectramax 190 microplate reader, the number of organoids per well was visually counted by optical microscopy (x10 magnification).

In contrast to the use of optical microscopy, the assay facilitated the identification of a subgroup of dead white cells within a viable organoid. Moreover, this technique enabled the viability of organoids seeded onto 3D culture scaffolds to be visualised on a large scale. **Fig. 6. 3** clearly illustrates the characteristic punctuated profile of the control organoids, which disappears (dead) in the infected macrophage as co-culture to intestinal organoids. Where the non-infected organoids appeared as a co-culture transparent, circular with a clear outline and buds in contrast to the infected organoids which appeared dark in colour, suffering from disintegration in the epithelial cell periphery with unclear outlines indicating dead or disrupted organoids.

Hence, MTT staining represents the fastest and easiest method for visualising cytotoxicity and primary intestinal organoid viability.

MTT reduction cold also is quantitatively measured from DMSO-solubilised organoids and a correlation could be observed between organoids per well and relative MTT reduction. Since geraniol would be tested for its therapeutic activity against organoid infection the viability of geraniol to the organoids was tested. The charts in **Fig. 6. 2. A** indicated that geraniol treatment resulted in significant organoid death at the doses $\geq$ 168 µM ($p < 0.0033$) whereas doses $\leq$ 84 µM did not ($p < 0.05$), compared to the mock treatment (0 µM geraniol). The solvent, DMSO for geraniol, did not cause

significant organoid death at the concentration up to 2668 µM (Fig. 6. 2 B). Hence, geraniol concentrations < 84 µM was considered to be safe in application to the intestinal organoids.

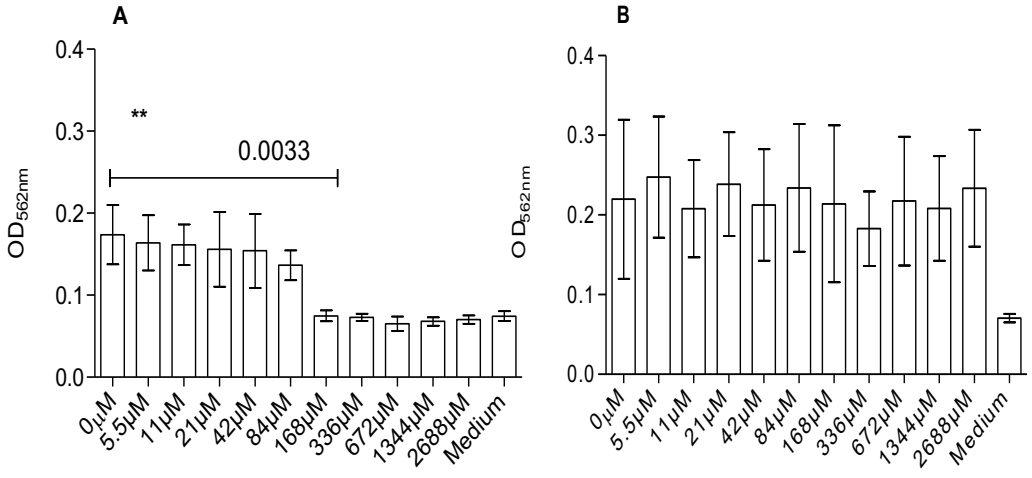


Figure 6. 2. Cell viability of geraniol and DMSO to the organoids. 5-6-day old organoids were treated with (A) geraniol and (B) DMSO overnight. MTT was added for one hour and the formation of formazan was measured at 562 nm absorbance. Dead and viable (out of 45-55 in total) organoids were scored under a microscope as illustrated in **Fig 6. 3** below. X-axes indicate the geraniol and DMSO concentrations, respectively, in A and B, and the Y-axes indicate the percentages of viable organoids per plate in A and B, respectively.

6. 3. 3. Intestinal organoid infection

6. 3. 3. 1. Co-culture of the organoids and infected Raw cells

It was known that AIEC translocates via M cells to infect underlining macrophages (Agus *et al.*, 2014; Etienne-Mesmin *et al.*, 2011). It was therefore of interest to know the impact of infected macrophages to cultured intestinal organoids, which would reflect the interactions between macrophages and the intestine. The murine Raw cell

monolayers were first infected with AIEC strain LF82 at MOI = 10 for 2 h. The intestinal organoid was then placed on top of the infected Raw cell monolayers and was observed for 7 days under the microscope. The organoids which were on uninfected Raws cells were bright, circular, transparent contains budds with clearly outlined peripheral whereas the organoids on top of infected Raw cells were dark with disintegrated peripheral without budds suggesting loss of structural integrity which means organoids death (Fig. 6.3).

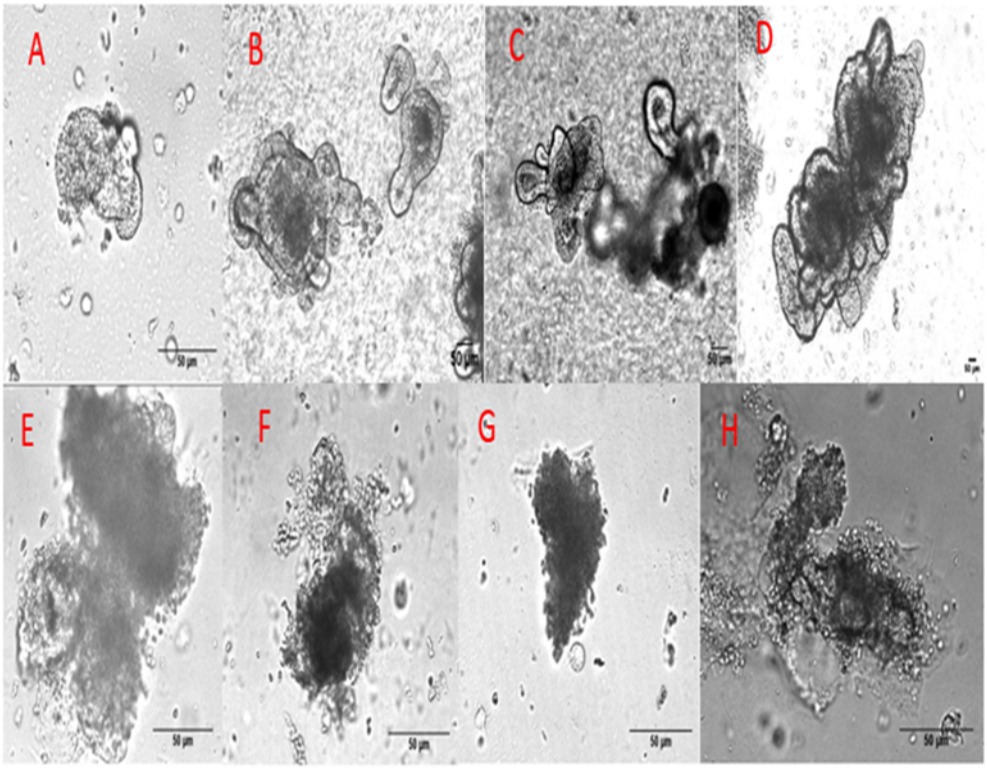


Figure 6. 3. Co-culture of organoids with infected murine Raw cells. The Raw macrophage cell monolayers were mock-infected with PBS or infected with AIEC strain (LF82) for 2 hr as per the gentamicin protection assay. Raw cells were washed extensively with PBS to remove extracellular bacteria, and organoids were placed on top of the mock-infected or LF82-infected raw cell monolayers 2-7 days. Bright-field images were taken using an epi-fluorescent inverted microscope. Co-culture of organoids with mock-infected Raw cells at

days 2, 4, 5, and 7 (A, B, C, D). Co-culture of organoids with LF82-infected Raw cells at days 2, 4, 5, and 7 (E, F, D, H). Scale bar = 50μm.

6. 3. 3. 2. Direct Infection

Next, the intestinal organoids were infected directly with the AIEC and *S. sonnei* strains. Ideally, the bacteria should be injected into the lumen of the organoids that reflect the natural site of infection (Forbester *et al.*, 2015; Zhang *et al.*, 2014). However, since no access to the injection facility was available, the bacteria were tried to be applied directly to the organoids grown in the Matrigel. This route of infection was at least relevant to *S.sonnei* that was known to invade epithelium via basolateral side rather than the apical side albeit AIEC was known to adhere via flagella and type I pili on the microvilli of the epithelium (Bringer *et al.*, 2007; Carayol & Tran Van Nhieu, 2013).

Approximately 70 – 85 organoids were infected with 10^7 CFU of *Shigella* or AIEC. The bacteria were incubated with the organoids for 30 min allowing for surface attachment. The organoids were then washed once with PBS to remove the extracellular bacteria, followed by a 60 min incubation in medium containing 50 μg gentamicin. Thereafter, 100 μg/ml MTT was added to the culture medium to distinguish live/dead organoids. Non-infected organoids turned dark rapidly, in a matter of minutes, after the addition of MTT (Fig. 6. 4. C, D). Infected-live organoids slowly became faint dark with a green background, presumably, GFP leaked from the bacteria which were transformed with PBS-erm GFP (Fig. 6. 4. A, B). Green bacteria were visible in Fig. 6. 4. A, B. The dead organoids due to infection turned dark slowly with no green background and completely lost structure (Fig. 6. 4. E, F)

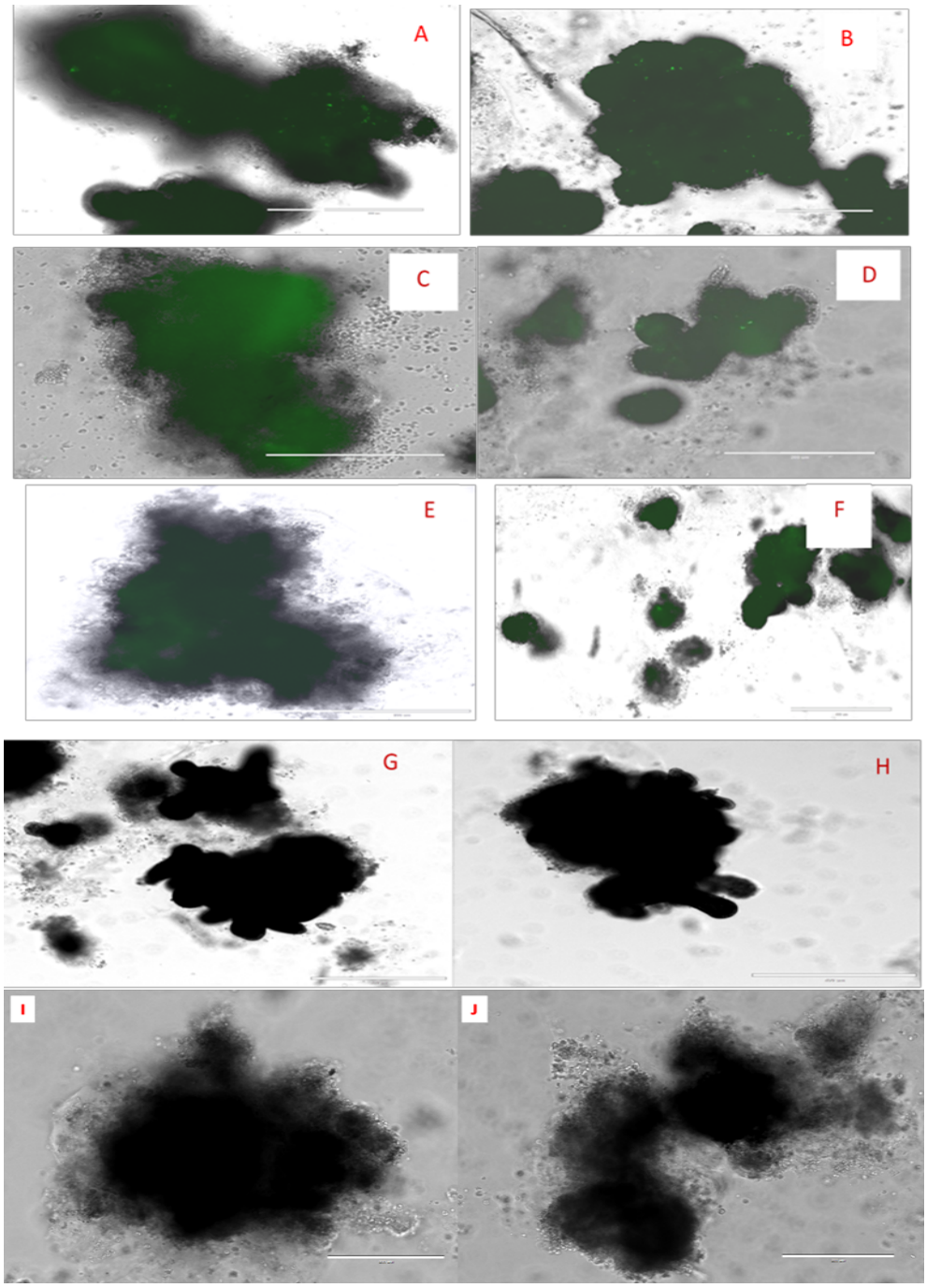


Figure 6. 4. Direct infection of organoids bacteria. (A, C, E) wild-type HM605, HH427, and *Shigella* infection of organoids imaged with 10X and 20X magnification; (B, D, F) organoid infection with *dsbA* mutant strains; (G, H) organoids without infection and (IJ) Infected organoids. Infected wild-type strain organoids showed morphological changes (e.g. disruption and unclear outline), while organoids infected with *dsbA* mutant strains showed

normal. Bacteria expressing GFP are represented by green colour. For each strain apart from the *dsbA* mutant *Shigella* strain, a scale bar of 200 µm was used, while an uninfected organoid scale of 400 µm was used. All experiments were conducted in triplicate. Likewise, all conditions were investigated thrice, with more than 65 organoids per sample.

The live/dead organoids were counted, and the results revealed that the *dsbA* mutant strains were associated with a higher number of live organoids compared to the respective wild type strains. This difference was statistically significant with $p < 0.0001$ (Chi-square and Mann Whitney test of Graph Pad Prism 5; Fig. 6. 5.).

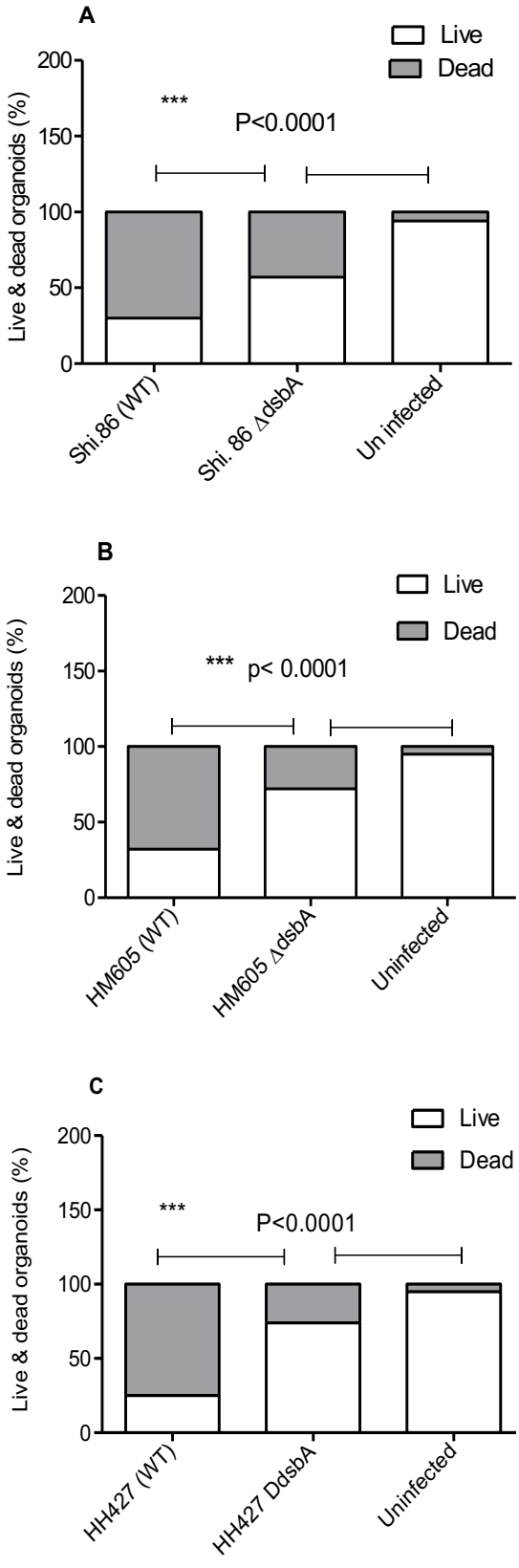


Figure 6. 5. The count of living/dead organoids. The values depict the average of three measurements ± standard deviation. In all cases, the wild type strain infection resulted in more dead organoids compared to the isogenic *dsbA* mutant strain (p < 0.0001 for all strains). Chi-square and Mann Whitney tests were performed using Pad Prism 5.

Since the green bacteria were visible in live organoids (Fig. 6. 4. A, B), the numbers of the intra-organoid bacteria were counted. This revealed that the AIEC *ΔdsbA* strain had significantly higher numbers than the wild type and the pJYu9 complemented strain (p = 0.0006; Fig. 6. 6. B). However, there was no difference between the wild type, the *dsbA* mutant, and the complemented *S. sonnei* strains (Fig. 6. 6. A).

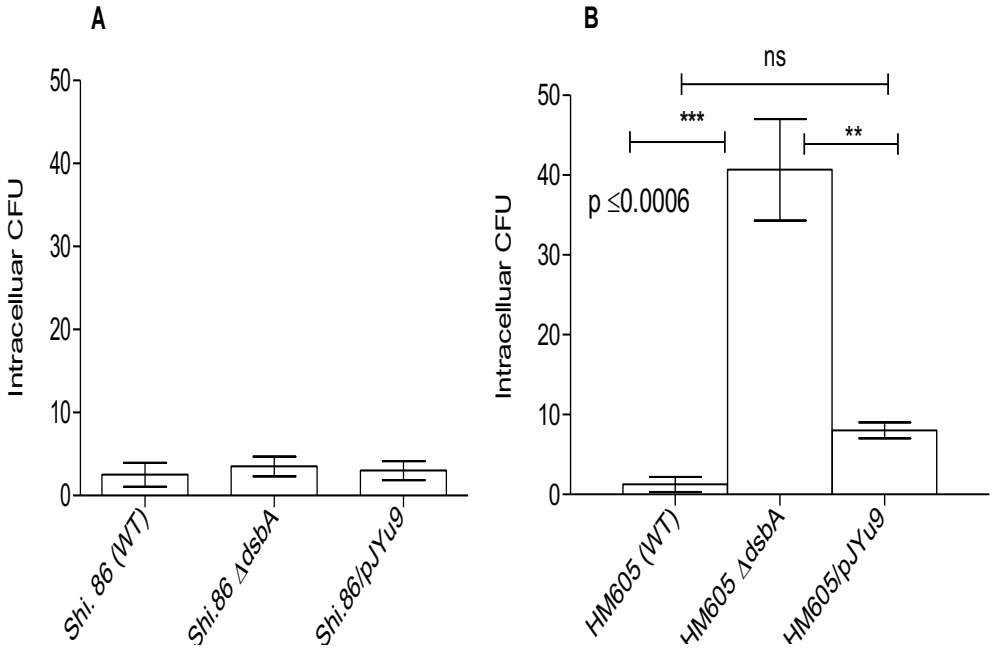


Figure 6. 6. Quantitation of intra-organoid bacteria. Organoids were infected with *Shigella sonnei* strains (A), and AIEC-MH605 strains (B), respectively. Each strain was examined in triplicate with multiple (> 10) organoids. Intestinal organoids were infected for 1 h; the organoid was re-cultured in condition media containing 50 μg gentamycin and incubated for a further 1 h. The data are means of three technical replicates for three biological replicates ± SEM. One-way Anova of Graph Pad Prism was used for statistical analysis.

6. 3. 4. Effect of geraniol and L-NAME on co-cultured organoids and infected macrophages

Because both microorganisms are intracellular this has been done in the same way as shown in Fig. 6. 3 except that geraniol (84µM) or L-NMAE (25mM) was added to the culture medium in addition to 50 µg gentamicin. Between 40 and 45 organoids/well were used for each of the bacterial strain and images were taken under epi-fluorescent inverted microscopy (Fig. 6. 7), and representative images were shown in Fig. 6. 7. In the presence of geraniol, all organoids looked normal where the organoids transparent, circular with clear budding and periphery (E, F, G) just like those mock-infected (A-D). In contrast, in the presence of L-NAME, all organoids appeared dead with complete disintegration in the periphery of the organoids without buds and surrounded by a transparent cavity (H, I, J). by counting live/dead organoids, geraniol treatment resulted in 83% of live organoids whereas L-NMAE treatment resulted in only 24% live organoids ($p < 0.0001$; Fig. 6. 8).

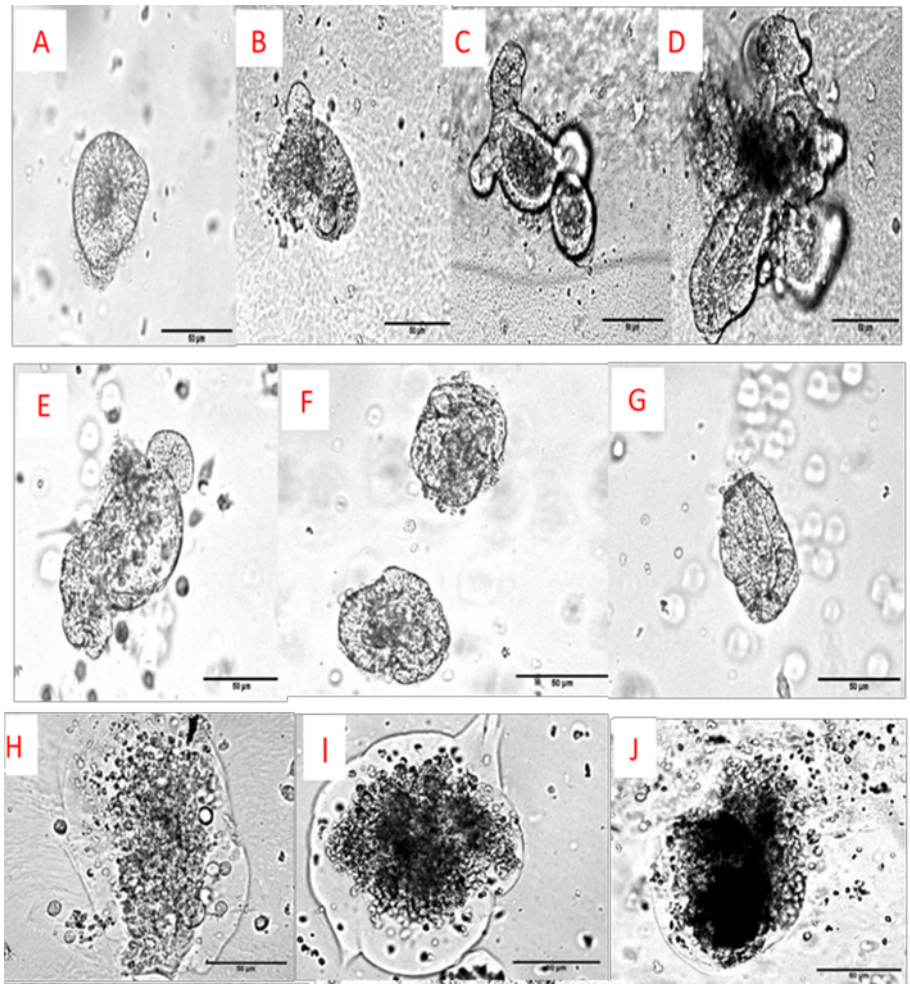


Figure 6. 7. Geraniol or L-NAME treatment of co-cultured organoids and infected Raw cells. Co-culture conditions were set the same as in Fig 6. 3. A to D, organoids were co-cultured without infection of Raw cell monolayers. Remainders were all co-cultured with Raw cell monolayers infected with LF82. E, F, G were supplemented with geraniol (84 µM/ml). H, I, J was supplemented with L-NAME (25 mM). Bright-field images were taken using epi-fluorescent inverted microscopy (scale bar = 50µm). (A-D):

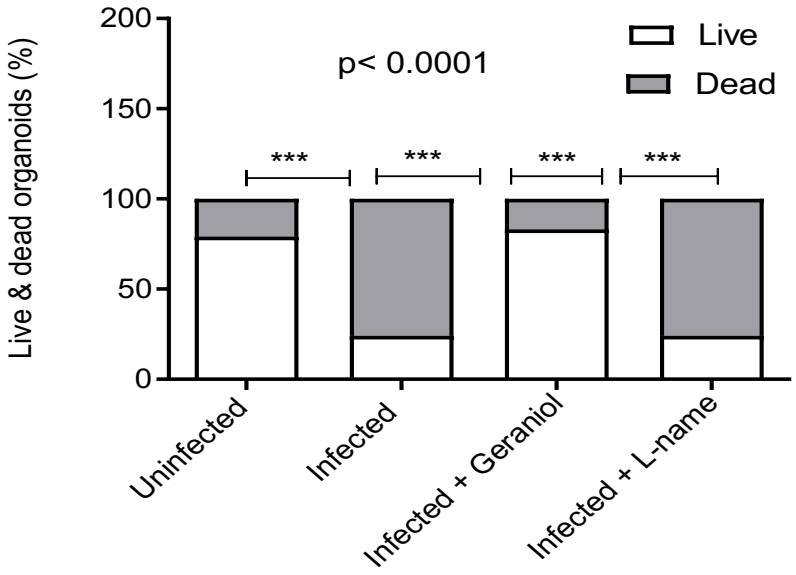


Figure 6. 8. Quantitative analysis of live/dead organoids as a result of geraniol or L-NAME treatment on organoids that were co-cultured with infected Raw cell monolayers. Data were generated from the same experiments shown in Fig 6. 7. 45-50 organoids were counted for each of the groups. A two-way ANOVA test of prism 5 was applied to determine significance between two groups. In all cases, *p*-value < 0.0001 was found indicating statistical significance.

6. 3. 5. Organoid treatment with inflammatory cytokines and LPS

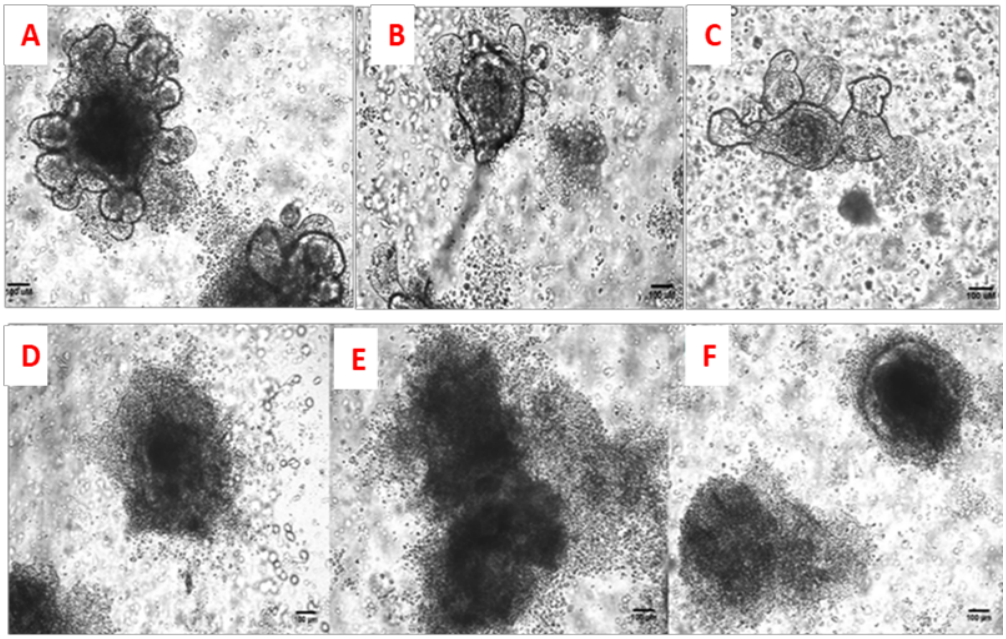


Figure 6. 9. Impact of TNF, IFN, and LPS to organoids. Organoids were either mock-treated (A, B, C) or treated with respective agents for two days: (D) IFN-γ; (F) TNF-α; (E) LPS of *E. coli*. Bright-field images were taken using epi-fluorescent microscopy (scale bar = 100 μm).

Organoids were also used to investigate the impact of inflammatory cytokines and LPS. Following the growth of organoids for three-four days (70 to 75 organoids/well; in 24 well plates), 10^5 macrophages were added and subjected to overnight incubation at 37°C with 5% CO_2 by using conditional mini gut media. Next day 50 ng IFN-γ, or 100 ng/ml TNF-α (Rauch *et al.*, 2017) or 1 μg LPS was added, which were permitted to pass into the culture. The treatment was allowed for one day. Each experiment was performed three times and the results revealed that all three agents caused significant deaths of the organoids, showing structuring disintegration with dark centers (Fig. 6. 9. D–F). In contrast, live untreated organoids were bright with

clear peripheral (Fig. 6. 9. A–C). By counting the live/dead organoids (Fig. 6. 10) it was found that the percentage of live non-treated organoids and organoid co-culture macrophages was 98% and 86%, respectively. Those treated with IFN-γ, TNF-α, and LPS the live organoids were 4%, 9%, and 10%, respectively ($p < 0.0001$). To reduce the subjectiveness, the organoids were also stained with trypan blue (Fig. 6. 11) and the counting of live/dead organoids revealed similar results (Fig. 6. 12).

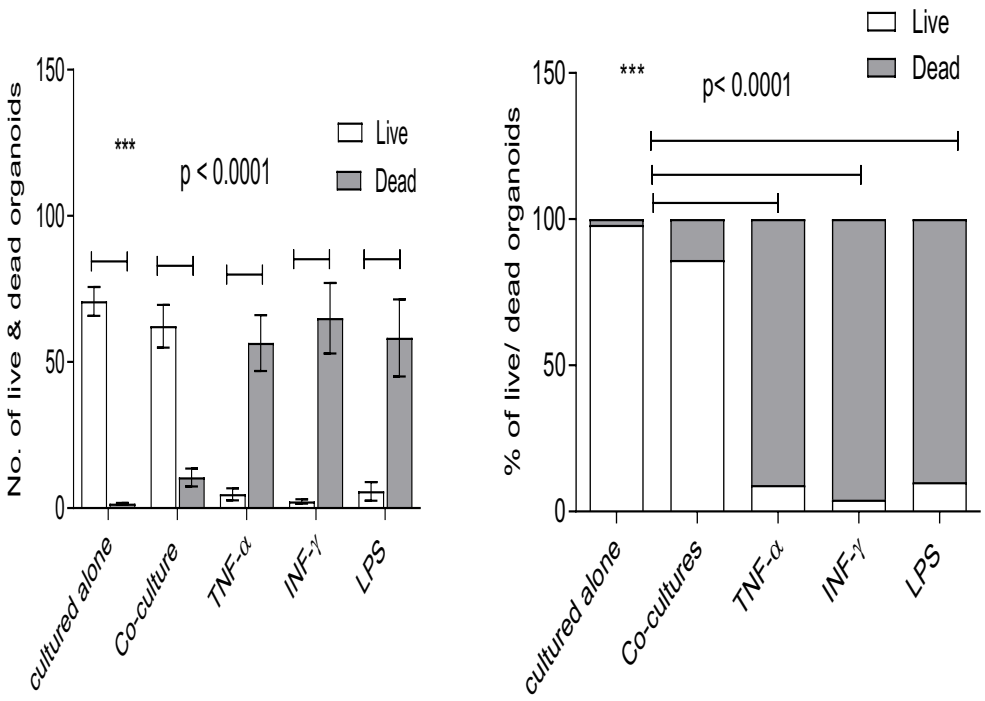


Figure 6. 10. Impact of TNF, IFN, and LPS on organoids that were co-cultured with Raw cells mock-infected or infected with strain LF82. Graphical illustration of live and dead organoids, macrophage co-cultures treated with cytokines, and LPS. The values represent the average of three measurements ± standard error. There is a significant difference between the bars with different letters ($P<0.0001$), by using two-way ANOVA and Chi-square of Graph Pad Prism 5. Here, X indicates the concentration of cytokines and LPS, whereas Y represents the number of live organoids per plate following treatment.

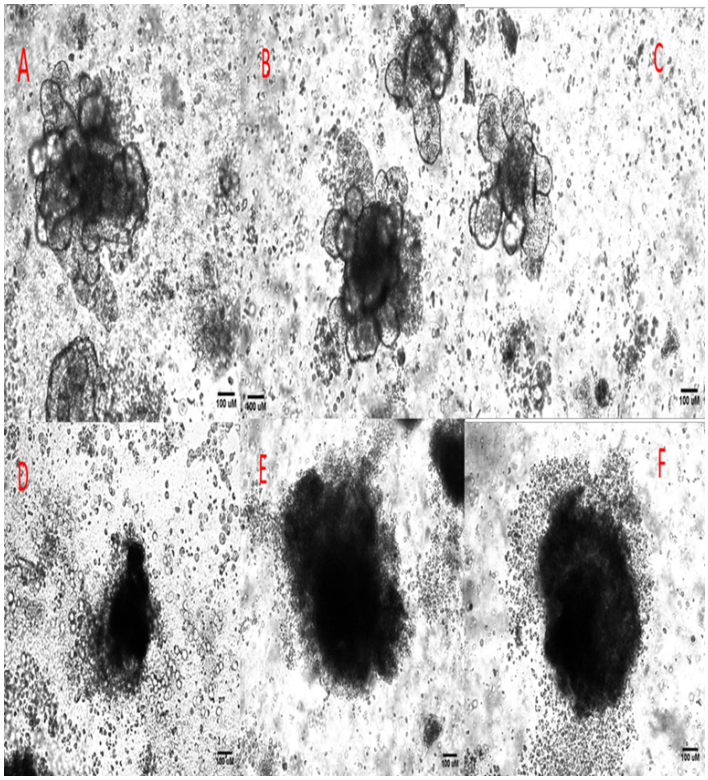


Figure 6. 11. Trypan blue staining of organoids, macrophages co-culture. (A to C): Normal organoids; Organoid treated with IFN-γ (D), LPS (E), TNF-α (F). Confocal epi-fluorescent microscopy (scale bar = 100 µM) was conducted 1-2 days after the cultures were exposed to cytokines in order to observe transformations in morphology.

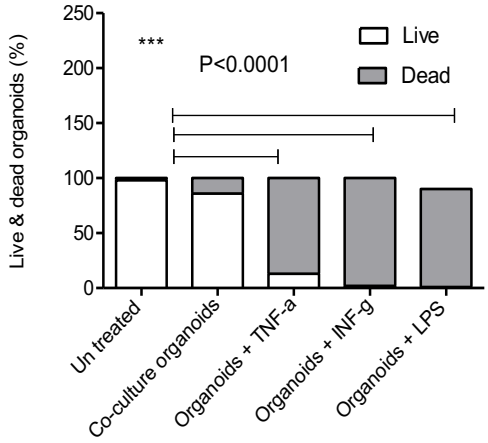


Figure 6. 12. Live/dead organoids counts. The macrophage-Raw cell co-cultures were stained with trypan blue. 70-75 total organoids were counted for each treatment and the experiments were repeated three times. Statistical analysis was done using Chi-square of Graph Pad Prism 5.

6. 4. Discussion

6. 4. 1. Challenging in culturing intestinal organoids

In this study, organoids have been successfully developed from 50 crypts per 50 µl of Matrigel droplet although there is a shortage in laboratory facilities such as incubators allocated for this type of culture, the cost of growth factors, and Matrigel. Because this is the first-time organoids grow in the laboratory as well, nobody has experience in this technique, so the researcher worked successfully without support.

The organoids showed an oval shape at 24 hours after cultivation, and at 48 hours they showed an elongated shape. At 72 hours, they started to develop buds that constituted the crypt structures. Not all organoids possessed the same number of buds. A major observation was that, compared to fraction purity, the number of crypts was of greater significance at the beginning of the culture because the crypt is very fragile easy to be broken. Shaking carefully by hand is, therefore, a major challenge in producing high-quality crypts, but not all crypts with full structure can grow. Organoids successfully developed from crypts, but there was some contamination of villi fragments that underwent apoptosis in a couple of days. Dead epithelial cells were eliminated during organoid passaging.

To determine whether organoid culturing was enhanced by reduction of mechanical stress during the separation of the crypts, the intestinal portions were pipetted with significant care during the washing procedure. crypt-enriched fractions were produced as a result of more careful pipetting, whereas Villi-enriched fractions were generated as a result of less careful pipetting. Consequently, the two types of fractions could be isolated more effectively, and therefore a purer crypt suspension for the commencement of organoid culture was obtained. The next step was to determine whether the organoid yield depended more on the amount or purity of crypts in a fraction. The number of crypts and the exact number occurring in a droplet may be different because precise identification between crypt structures and certain villi portions is challenging.

6. 4. 2. MTT geraniol analysis in the intestinal organoid

The present work highlighted a crucial adaptation of the MTT assay, enabling both qualitative and quantitative examination of organoid viability. Qualitative results were acquired by optical microscopy following *in-situ* MTT conversion, while SDS treatment of Matrigel, followed by dissolving in DMSO, enabled the acquisition of quantitative results via absorbance measurement. Notably, the results indicated that, with high doses (starting from 168 -2688µM) of geraniol, the death of organoids took place at higher rates when compared to the use of low doses (starting from 5.5-84µM). In the case of low doses, these led to viable organoids (Figure. 6. 2). Furthermore, the present results are in agreement with previous work by Grabinger *et al.* (2014) and Young and Reed (2016) in using MTT to evaluate the viability of organoids.

Noteworthily, this study's novel application of *ex vivo* cultured intestinal organoids, as well as an adapted MTT assay, could furnish a fascinating and economical substitute to *in vivo* animal experimentation involving intestinal epithelium-damaging compounds. From these findings and the NO assay, as well as based on the determination of the efficacy of geraniol via gentamicin killing assay, this study used 84 µM concentration of geraniol to complete other experiments that correlated in the treatment of AIEC-infected macrophages as a co-culture with organoids (see Figure 6. 2A and refer to Chapter 4's NO experiments). Additionally, this dose has been found to affect bacteria and has no effect on organoids. Moreover, the study's results revealed no effect of DMSO when used in all the experiments, as shown in Figure 6. 2B. DMSO was used to dissolve the geraniol because it is water or media insoluble.

6. 4. 3. Infection with intestinal organoids

6. 4. 3. 1. Co-culture infection

AIEC has been known to translocate to infect underlining macrophages via M cells (Agus *et al.*, 2014; Etienne-Mesmin *et al.*, 2011). Therefore, knowing the impact of infected macrophages on cultured intestinal organoids was of interest, which would reflect the interactions between the macrophages and the intestines.

The results obtained revealed that non-infected (Figure 6. 3A-D) and infected organoids (Figure 6. 3E-H) differed significantly, corroborating the findings of (Wilson *et al.*, 2015) and (Rookmaaker *et al.*, 2015). Upon invading the enteroid epithelial cells, the bacteria corrupted the peripheral of organoids leading to loss of structural integrity, causing the epithelial cells to break down and eventually die. Moreover, as reported in the studies conducted by Zhang *et al.* (2014) and Leslie and Young (2016), it was observed that the NF-kB pro-inflammatory response was activated in the infected epithelium. The possibility of killing organoids in this experiment may be because, first of all, bacteria will activate some of the non-infected macrophages found in the medium leading to the direct killing of organoids. Secondly, activated macrophages could result in the production of cytokines such as TNF-α, IL8, and IL6 which lead to organoids being attacked and causing death. Finally, replication of bacteria inside the macrophages (Bringer *et al.*, 2006a) would eventually lead to macrophage rupture resulting in the release of the bacteria that attacked the organoids and caused death.

These findings encouraged the researcher to complete and understand the role of cytokines in the ulcer formation of CD in the next experiment.

Studies found that seeding macrophages along the basal surface of these monolayer cultures, barrier properties, including epithelial cell height and transepithelial electrical resistance, were enhanced, indicating a strengthened barrier upon co-culture with macrophages (Noel *et al.*, 2017). Moreover, when pathogenic strains of *E. coli* were introduced to the apical surface of the co-cultures, macrophages were able to sense the pathogen and evoke phagocytic Defense machinery. In addition,

macrophages underwent morphological changes in response to the pathogenic *E. coli*, extending projections across the epithelial barrier in order to survey the luminal environment.

6. 4. 3. 2. Direct infection

Earlier studies have addressed the part played by *dsbA* in pathogenic *E. coli* virulence. Dailey and Berg (1993) reported that *dsbA* participated in the biogenesis of flagella and other appendages on the surface of bacterial cells, while Donnenberg *et al.* (1997) and Jacob-Dubuisson *et al.* (1994) indicated that *dsbA* was implicated in the development of fimbriae in EPEC and UPEC. Likewise, Okamoto *et al.* (2001) identified *dsbA* as playing a key role in the biogenesis of toxins (e.g. heat-labile and heat-stable ETEC toxins). Furthermore, Bringer *et al.* (2007) observed that, in LF82, replication of a *ΔdsbA* mutant in macrophages is impossible, and additionally, that this mutant has trouble attaching to and invading intestine-407 epithelial cells. Therefore, the researchers concluded that more than one virulence function in the AIEC strain LF82 was dependent on *dsbA*. The researchers also noted that, in the context of *in vitro* growth of LF82 in a medium partially replicating phagolysosome conditions, there was upregulation of *dsbA* gene transcription, comparable to the *htrA* gene. This implied that the activation of *dsbA* occurred in phagolysosomal conditions. Hence, the literature indicates that LF82 relies on *dsbA* oxidoreductase to withstand eradication in unfavourable phagosome conditions. Similar to the LF82 *dsbA* mutant, an *S. flexneri ΔdsbA* mutant was found to display poor growth in macrophages (Yu *et al.*, 2001).

The intestinal organoids were then directly infected with the strains AIEC and *Shigella sonnei*. Ideally, the bacteria should be injected into the organoids 'lumen reflecting the infection's natural site (Forbester *et al.*, 2015; Zhang *et al.*, 2014). Since there was no access to the injection facility, it was attempted to apply the bacteria directly to the organoids grown in the Matrigel. This infection route was at least relevant to *S.*

sonnei, which was known to invade epithelium via the basolateral side rather than the apical side, although AIEC was known to adhere to the epithelium microvilli via flagella and type I pili (Bringer *et al.*, 2007; Carayol & Tran Van Nhieu, 2013).

Based on the above, this study investigated differences among wild type, *ΔdsbA* mutant, and transformed pJYu9 plasmid strains in terms of direct organoid infection at MOI 10^7, as shown in Figure 6. 4, 6. 5 and Figure 6. 6. To the best of our knowledge, no previous study has adapted an organoid model to determine how intestinal organoids of crypt origin responded to *Shigella* and AIEC (HH427 and HM605) strains.

The findings of this work revealed that organoids infected with *Shigella* and AIEC (wild type and transformed pJYu9 plasmid) exhibited significant structural disruption, with cell breakdown (Figure 6. 4 ABEF) resulting in deformation and eventual death of organoids. The live/dead organoids were counted, and the results showed that the *dsbA* mutant strains were associated with a higher number of live organoids compared with the respective wild type strains (Figure 6. 5 ABC), where this difference was statistically significant with $p<0.0001$. Two approaches were used to demonstrate this. The first approach was to employ MTT staining and Evo's microscope to quantify the invading bacteria (Figure 6. 6 AB), which showed that the different *Shigella* strains did not differ significantly from one another. It was concluded that most *Shigella ΔdsbA* mutant strains are unable to penetrate or invade the organoids and remain outside in the medium and are killed by gentamicin as opposed to the wild type strains that can invade and replicate within the organoids, as a result, will result in rupture and kill the organoids; and release the bacteria into the medium and kill them with gentamicin. This explains why there were no significant differences in the number of bacteria counted between *dsbA* mutant and wild type strains (Figure 6. 6 AB). This study again highlighted the importance of the *dsbA* gene in *Shigella* virulence and pathogenesis. It would be recommended to further investigate the bacterial invasion within the organoids using a histopathological examination.

Or the *Shigella ΔdsbA* mutant strain was still virulent for this model. What this seems to imply is that, even when mutated with the *dsbA* gene, *Shigella* retains virulence,

or else the bacteria themselves trigger an inflammatory factor from organoids (e.g. TNF or NO_2), which subsequently affects organoid structure and survival. This was consistent with the findings relating to biofilm development in this work also those relating to cell line and larval infection reported earlier by Mahmoud *et al.* (2016). Further study is needed to clarify this aspect.

The organoids infected with the wild type and transformed pJYu9 strains of AIEC were severely damaged and lacked a clear outline (Figure 6. 4 A-H), whereas those infected with the Δ*dsbA* strain showed no structural differences compared to normal organoids. To demonstrate this, the bacterial invasions were quantified, with a significantly higher number of invading wild type and transformed pJYu9 plasmid strains than Δ*dsbA* mutated strains (p<0.0006 (Figure 6. 6). Using pJYu9 in Δ*dsbA* mutated strain, the importance of this gene in the virulence and pathogenicity of the CD-AIEC strains was demonstrated, with this strain having the same impact on organoids as the wild type -HM605 strains. The use of pJYu9 in the Δ*dsbA* mutated strain proved how important this gene was for CD-AIEC strain virulence and pathogenicity, with this strain having the same impact on organoids as the wild type HM605 strains.

Since the green bacteria appear in live organoids, the second approach has been adopted to demonstrate the structural similarity between organoids infected with mutant strains and normal organoids. This revealed that the *Shigella* strains did not differ significantly (Figure 6. 6A), but AIEC-HM605 and Δ*dsbA* mutant strains did (Figure 6. 6B). Furthermore, the bacterial count on the well/plates of wild type and transformed pJYu9 plasmid strains did not differ significantly. This indicated that organoid structure and budding were unaffected, despite the high count of Δ*dsbA* mutated strains invading the organoids. What this implies is that, in the absence of the *dsbA* gene, the bacteria lose their virulence to epithelial cells, regardless of additional virulence factors. This was confirmed not only by raw macrophage and larval infections but also by biofilm development. This signifies that the *dsbA* gene is a vital factor in bacterial virulence, pathogenesis, and organoid invasion.

Drawing on the study conducted by Karve *et al.* (2017), it may be argued that the *Shigella*-based mutant still virulent for this model may be due to *Shigella* retaining virulence other than the *dsbA* gene or polarised organoid trigger factor leading to death, whereas the AIEC (HM605)-based mutant strains behaved in the intestine similarly to non-pathogenic. In other words, organoids recognise the bacteria based on the nature of the latter in the environment. This supports the hypothesis that the wild type strain of *E. coli*, which has pathogenicity for eukaryotic cells, is transformed through contact with normal flora. Another possibility is that, as *dsbA* mutants do not kill organoids, they are protected within the organoids; whereas the wild type destroyed the organoids, and therefore gentamicin killed the bacteria, as in the case of AIEC.

This was what primarily motivated the selection of the *dsbA* gene for the purposes of the present study, irrespective of other research investigating the same bacterium and gene, with comparative analysis using other diarrhoea-causing pathogenic bacteria (e.g. *Shigella* spp). Further research is needed to investigate the impact of the *dsbA* gene associated with other pathogenic bacteria, based on an organoid model. It is also worth emphasising that these results were consistent with those reported on biofilm formation, macrophages, and larval infections.

6. 4. 4. Geraniol and L-NAME infected macrophages and organoid treatment

The purpose of the experimental work conducted in this study was to determine the efficiency or efficacy of terpenoids, especially geraniol, as a treatment method for infections by Gram-negative bacteria (adherent invasive *E. coli*), given their ability to deactivate rather than destroy bacteria.

Unlike L-NAME, geraniol conferred protection on organoids against the infected macrophages or cytokines released directly or indirectly by AIEC (Figure 6. 7E-G). This indicated that geraniol had antibacterial effects, disrupting the virulence markers of AIEC within macrophages by hindering the development of disulphide bonds in the

Dsb protein (see Chapter 1). Quantification of live and dead organoids with the use of a microscope confirmed this, revealing that geraniol-treated live organoids numbered more than dead organoids compared to those treated with L-NAME or not treated at all (Figure 6. 8). This would suggest that geraniol is necessary to inhibit the growth of intracellular bacteria to keep organoids alive; whereas L-name inhibition of the production of nitric oxide is not enough to save organoids (Figure 6. 7), which implies that geraniol aids macrophages in targeting bacteria compared to control (non-treated cells). Several studies have reported consistent results (Daisy *et al.*, 2008; Halili *et al.*, 2015; Ireland *et al.*, 2014). At the same time, through the use of geraniol, beneficial bacteria were spared, and bacterial resistance avoided (Astashkina & Grainger, 2014; Liu *et al.*, 2016; Vela & Chen, 2015).

Using high doses of geraniol caused toxicity to the cell organoids (see Figure 6.2A-B). For this reason, 84µM was selected as the main dose to treat the organoids. This explains why the number of organoids co-cultured with macrophage and treated with this dose was lower than that of non-treated organoids, which served as a control. According to the literature, geraniol has a statistic effect because of the activity, which is correlated with the deactivation of the bacteria rather than killing them. Furthermore, this depends on the concentration (see Chapter 1 for a discussion of the geraniol's mechanism of action). Here, the last point to emphasise is that the rationale for selecting this dose stemmed from the context of the present inquiry. In particular, the experimental work presented here was a postscript to a pair of other student-led projects, which were administering a similar dose. In turn, the author's supervisor and student colleagues played a role in defining the dosage.

Interestingly, studies were found that geraniol inhibits, both *in vitro* and *in vivo*, the expression of cyclooxygenase-2 (COX-2), an enzyme that plays an essential role in the inflammation and targeting of NF-kB (Chaudhary *et al.*, 2013; Medicherla *et al.*, 2015; Strillacci *et al.*, 2010). Moreover, It has been shown that geraniol stimulates the production of interleukin 10 (IL-10) in macrophages (Murbach Teles Andrade *et al.*, 2014). In this context, it has inflammatory suppressive action, which is beneficial for the treatment of hyperinflammatory shigellosis (Mirza *et al.*, 2018), as well as Crohn's disease. Loss of IL-10 signalling induces microbial hyper-responsiveness and over-

production of PGE2 in tissue macrophages (Mϕs), which in turn restricts their microbicidal capacity. This toxic combination of hyperactive Mϕs and reduced bacterial clearance drives chronic intestinal inflammation as a result of deficiencies in IL-10/IL-10R (Mukhopadhyay *et al.*, 2019).

To the best of our knowledge, this study is the first to report on the use of an organoid model for assessing the efficacy of geraniol as an antibacterial agent against AIEC. The success of the model could lead to the use of the same technique to identify the activities of other antibiotics or compounds.

6. 4. 5. Organoid therapy with different agents

The related experimental work sought to investigate the involvement of cytokines in ulcer development through their role in damaging intestinal epithelial cells, specifically through the excessive stimulation of the immune system (Neurath, 2014). IFN-γ and TNF-α were selected in the present study because these cytokines were the first to be secreted by macrophage during the infection occurrence (see this chapter's introduction as well as Chapter 1). Elevated levels of TNF are associated with cytokines IFN-α, IL17, and IL-12 (Fujino *et al.*, 2003) in CD and ulcerative colitis, diarrhoea, mucosal ulceration, and intestinal bleeding. However, the direct effects on epithelial cells of these inflammatory molecules remain uncertain (Bradford *et al.*, 2017).

In the present work, the organoid was used to investigate the impact of using cytokines and LPS. After organoid growth, macrophages were added and incubated overnight at 37 °C with a carbon dioxide content of 5%. Subsequently, 50 ng IFN-γ, 100 ng/ml TNF-α, or 1 µg LPS were introduced, which were allowed to pass into the culture. Meanwhile, the same concentration of cytokines and LPS was used to stimulate the organoids alone (directly), which did not have any effect (Rauch *et al.*, 2017).

The study observed that organoids sustained massive structural damage, and afterward died in great numbers, when they were stimulated with IFN-γ, TNF-α, and LPS co-cultured. The apoptosis-inducing effect undoubtedly exists in terms of the concentration used in the experiment (Figure 6. 9 D-F). This is because cytokines such as TNF inhibit normal intestinal epithelial morphogenesis, which can lead to the formation of tight epithelial monolayers, and instead facilitate an alternative mode of intestinal epithelial morphogenesis by inducing apoptosis. In turn, this results in the formation of "leaky" monolayers and organoid death. This finding was consistent with other studies (Gunther *et al.*, 2015; Juuti-Uusitalo *et al.*, 2011; Rauch *et al.*, 2017; Singh *et al.*, 2016; Viard-Leveugle *et al.*, 2013).

The above cytokines were selected because they are excessively secreted from T helper 1 (Th1) cells in CD, which plays an integral role in pathogenesis. The secretion of these cytokines is augmented by the secretion of the same proinflammatory cytokines from macrophages in CD. Such cytokines mediate a number of biological effects that potentiate the immune response, which can result in oedema in the propria lamina and consequent breaks in the epithelial monolayer (Carpenter & Talley, 2000). However, these cytokines may directly target the intestinal epithelial cells to generate signalling pathways that promote apoptosis and/or inhibit the function of tight junctions, both of which may result in reduced epithelial integrity (Schulzke *et al.*, 2009; Turner, 2006).

From the above, it seemed reasonable to conclude that the infection with *Shigella*, in case of shigellosis, or AIEC, in the case of CD, will lead to the activation Th1 cells, which subsequently activates macrophages to act in one of two ways: firstly, to produce cytokines such as IFN-γ and TNF-α, which stimulates the release of other cytokines (e.g. IL-2), thereby resulting in hyper inflammation in the GIT for both diseases; or secondly, to attack the bacterial invader, thereby leading to granulomatous inflammation (as in the case of chronic CD). All these results were consistent with macrophages, and larval infections.

From this perspective, the study contributed to existing evidence that the epithelium was adversely affected and underwent numerous transformations when the immune

system (macrophages) was excessively stimulated in the context of bacterial infections. Also, the results suggest that the secretion of cytokines is carried out by macrophages and has nothing to do with organoids (supporting data are not given in this manuscript). Furthermore, by highlighting the intensified release of inflammatory cytokines and the manifestation of other inflammatory reactions, the study contributed to knowledge surrounding the development of ulcers due to CD. Confirmation of the above results was performed by counting the dead and live organoids among those with and without treatment, revealing that more cytokine-treated organoids died compared to non-treated organoids, as well as the same number as the LPS-treated positive control (Figure 6. 10). The use of trypan blue to stain the organoids before counting the dead and live ones reinforced the results (Figure 6. 11 and 6. 12).

6. 5. Conclusion

At the outset of this chapter's discussion, the steps needed for culturing intestinal organoids were outlined, with a particular focus on specific challenges.

Following this, the study highlighted a crucial adaptation of the MTT assay, enabling both qualitative and quantitative examination of organoid viability. The assay correlated well with the authentic and microscopically identified quantity of dead and viable crypts or organoids in culture over a wide range of dilutions. Results were reported that were consistent with the extant and related literature, particularly in terms of the novel application of *ex vivo* cultured intestinal organoids and an adapted MTT assay to create a sensitive and facile protocol for the screening of treatments and drugs with respect to toxic and cell death-inducing properties.

Noteworthily, one of the experiments conducted in this chapter, which involved investigating differences among wild type, *ΔdsbA* mutant, and transformed pJYu9 plasmid strains in terms of direct organoid infection, is the first published in the

literature, and the findings revealed that organoids infected with *Shigella* and AIEC (both wild type and transformed pJYu9 plasmid) exhibited significant structural disruption, with cell breakdown resulting in deformation and eventual organoid death. Significantly, the findings contradicted others reported in the literature, as well as those reported in this study relating to biofilm development. Other results implied that, without the *dsbA* gene, bacteria lose their virulence to epithelial cells, irrespective of additional virulence factors. Hence, it is reasonable to conclude that the *dsbA* gene is a vital factor in bacterial virulence, pathogenesis, and organoid invasion.

The experimental results also indicated that dissimilar to L-NAME, geraniol conferred protection on organoids against the infected macrophages or cytokines released directly or indirectly by AIEC, attesting to the antibacterial effects of geraniol and its ability to aid macrophages in targeting bacteria. Furthermore, through the use of a reasonable dose of geraniol (in this case, 84μM), beneficial bacteria were spared, and bacterial resistance avoided, which was consistent with the results reported elsewhere in the literature. At the same time, the experimental work sought to examine the effect of cytokines IFN-γ and TNF-α on ulcer development, specifically through their role in damaging intestinal epithelial cells.

6. 6. Recommendation

Further study would be recommended on:

- Second cross over of AIEC with Pcp20.
- T6SS of AIEC and cell line invasion and proliferation.
- OmpA of AIEC and cell infection.
- Organoids infection to *dsbA* mutant of *Shigella.*
- Organoids for secretion cytokines.
- Histopathological studies for direct or co-culture infected organoids and organoids stimulated with cytokines.
- Role of *proQ* gene in the pathogenesis of *Shigella*.

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