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Understanding *Salmonella enterica* serovar Dublin as a cause of abortion in cattle

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Abstract

Salmonella enterica serovar Dublin (*S.* Dublin) is a host-adapted non-typhoidal *Salmonella* serovar associated with disease predominantly in cattle as well as a variety of other species including humans. *S.* Dublin is one of the most common infectious causes of bovine abortion in the UK and is the most commonly isolated *Salmonella* serovar from cattle. *S.* Dublin can persist in the environment and reside in sub-clinically infected animals, making it extremely difficult to eradicate on farm. Infection in cattle is acquired via the faecal oral route, and the bacterium can be shed in faeces, urine and milk. As a zoonotic disease, *S.* Dublin also poses a risk to human health and food security, with many cases in humans being found to be associated with consumption of contaminated dairy and beef products. Additionally, an abortion in a dairy herd in the UK is estimated to cost upwards of £630 (Cabell, 2007).

Compared to serovars like *S.* Typhimurium and *S.* Paratyphi, *S.* Dublin is a relatively under-researched serovar. Little is known about its virulence in host specific tissues and niches and the process of infection and dissemination in cattle is unknown. Furthermore, research into *S.* Dublin infection as a cause of abortion is scarce, with the host response to the pathogen during pregnancy currently unknown.

16 *S.* Dublin isolates from cases of clinical disease in cattle in the UK were phenotypically characterised for their growth and virulence in the Bovine Caruncular Epithelial cell line (BCECs). The comparison of the whole genome sequences of these 16 isolates to those of 250 other *S.* Dublin strains from the UK isolated between 2001 and 2019 from various origins of isolation confirmed that these 16 isolates were representative of the circulating population. Virulence factors were identified in the 266 total *S.* Dublin isolates using bioinformatic analysis, and a phylogenetic alignment of the accessory genomes of these isolates revealed distinct clustering of isolates from similar origins of isolation.

The survival of 4 of the 16 *S.* Dublin isolates was assessed in fresh bovine whole blood, and infection of BCECs was carried out to understand the expression of CXCL8, TNFα and PGE_2 via qPCR and ELISA. *S*. Dublin were able to survive in bovine blood for up to 2 hours and invaded and persisted in BCECs for up to 24 hours. BCECs up-regulated expression of CXCL8 and TNFα mRNA and PGE_2 in response to infection with *S.* Dublin over the course of 24 hours.

The work presented here is the first of its kind to identify the virulence factors of a large cohort of *S.* Dublin isolates and detail the survival of *S.* Dublin in bovine blood. Additionally, this is the first time that *S.* Dublin have been shown to infect cells from the placentomes of pregnant cattle and the first time that the host response to infection with *S.* Dublin has been investigated in a placentome-derived cell line.

Acknowledgements

I would like to take this opportunity to give my heartfelt thanks to a number of people whose support has been invaluable over the course of my PhD.

First and foremost, I would like to thank my supervisor, Dr Sabine Tötemeyer, for her unwavering support and friendship through what has been an incredibly challenging four and a half years. Sabine, you have been the best supervisor and mentor I could have hoped for and an even better friend. I am forever grateful to you.

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To my partner Haydn, thank you for being by my side through some of the most challenging times of my life so far. You make me laugh like nobody else can and have been the best shoulder to cry on

through my qPCRs not working or when write-up turned out to be one of the hardest parts of doing a PhD. I am so lucky to have you in my life and am so grateful to you.

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Thank you to my incredible parents, Jacque and Chris, who have always encouraged me to pursue my dream of doing a PhD and only sometimes told me not to do all of the society things I wanted less I get too distracted. I am so fortunate to have you both and I hope I have made you proud.

Finally - an odd acknowledgement but an important one nonetheless – thank you to the community of Sutton Bonington. SB has been my home for the best part of eight years and in a way, it always will be. The highs and lows during my university experience have been immeasurable, and the memories I take away with me are ones I will cherish for the rest of my life.

Acknowledgement of contribution to data collection and processing

Adam Blanchard conducted bioinformatic analysis of the *S.* Dublin and *S.* Typhimurium isolates including producing phylogenetic trees of *S.* Dublin isolates and running the sequence analyses to identify virulence factors and antimicrobial resistance genes in isolates of both serovars investigated.

Downstream data cleansing of the *S.* Dublin antimicrobial resistance genes from human and livestock isolates as well as functional categorisation of the genes identified was completed by 3rd year Veterinary medicine student Lucy Newman.

Downstream data cleansing of the *S.* Typhimurium virulence genes from all 266 isolates as well as functional categorisation of virulence genes which were not identified in *S.* Dublin isolates was completed by 2nd year Animal Science student Sophia Lewin.

Phenotypic antimicrobial susceptibility of 16 *S.* Dublin isolates and gentamycin sensitivity testing was conducted by 3rd year Veterinary Medicine student Stijn Brussen.

Dedication

I would like to dedicate this work to my wonderful parents, Jacque and Chris.

Despite everything, you were absolutely right – I really did have the best time at SB.

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COVID-19 Impact Statement

The COVID-19 pandemic impacted my PhD in a number of ways.

First and foremost, I was unable to undertake laboratory work from the end of April 2020 (when I returned to the university full time after finishing my industrial placement) to the beginning of August 2020 because of the government and university rules around laboratory occupancy. When laboratory access was granted, we worked under a rota system to keep the number of people sharing the space to a minimum. This also included a reduction in the number of hours in which we were allowed to undertake laboratory work and an enforced lunch break which interrupted longer experiments. This also meant I was often limited in the number of longer molecular procedures I could complete within a single day, considerably extending the period of time which was necessary to do the downstream processing required to investigate the host response to infection. The first experiments I conducted after I was granted access to the laboratory again involved culturing the Bovine Caruncular Epithelial Cell line (BCECs) which could take up to six weeks to grow sufficiently for use in experiments. Having finished portions of lab work and written everything up before going on placement, returning to the lab was essentially starting from scratch, as we had planned to start more complex infection experiments when I came back to study full time. It was therefore not feasible to complete any other lab work alongside culturing the cells as all other lab work was finished for prior chapters. This was also complicated by the national shortages of filtered pipette tips which were essential for the molecular work around exploring the host response to infection.

I received a six week extension to my stipend from the university but stopped receiving a stipend in November 2021. As a result, I had to borrow money from my family and eventually seek part-time employment to remain financially stable. My mode of study changed on the 31st January 2022 from full time to part time.

The considerable uncertainty around my PhD, alterations to my PhD project, financial instability and general pandemic uncertainty had a significant impact on my mental health. I experienced overwhelming anxiety and stress, feelings of hopelessness and both emotional and physical fatigue, particularly during 2020 and early 2021. This impacted my ability to conduct laboratory work and interrupted my sleeping and eating patterns which left me exhausted and with nausea and stomach pain. These feelings continued throughout the remainder of my PhD, during which time I experienced burn-out, stress and anxiety as well as depressive episodes. I had panic attacks because of the overwhelming stress and these occurred several times in the laboratory. I tried to access counselling from the university to try and learn new strategies to cope and spoke regularly to a DTP Welfare Officer from May 2021 until handing in my thesis.

Chapter 1 - General Introduction

1.1 - *Salmonella* spp

Salmonella spp. are gram-negative, facultative anaerobic, rod-shaped Enterobacteriaceae, comprised of around 2,500 serovars divided into two species – *Salmonella bongori* and *Salmonella enterica. S. enterica* is further categorised into six subspecies – *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* – by phylogeny and by the presence or absence of surface O (somatic), K (capsular) and H (flagellar) antigens under the White-Kuffman-Le Minor Scheme (Grimont and Weill, 2006). *S. enterica* and *salamae* are usually associated with warm-blooded animal infection including humans, whilst *arizonae, diarizonae, houtenae* and *indica* are associated with cold-blooded species (Brenner et al., 2000). *S. enterica* is the most common subspecies isolated in human infections, accounting for around 99% of isolations (Brenner et al., 2000; Chen et al., 2013).

Salmonella enterica subspecies *enterica* serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi) cause typhoid, or enteric fever in humans, usually resulting in diarrhoeal illness, pyrexia, and malaise (Basnyat et al., 2021). Without treatment, these typhoidal infections can be fatal and are more common in children in countries with poor sanitation and access to clean drinking water (Crump et al., 2015). In 2017, enteric fever was estimated to have caused 135,900 deaths amongst 14.3 million cases worldwide (Stanaway, Reiner, et al., 2019). Non-typhoidal salmonellae infections usually cause self-limiting diarrhoeal disease and are less frequently associated with systemic disease and death in humans (Chen et al., 2013). However, these non-typhoidal serovars can cause invasive disease which in 2017 caused an estimated 77,500 deaths in 535,000 cases globally,

a higher proportion of fatalities compared to typhoidal infections (Stanaway, Parisi, et al., 2019). Non-typhoidal salmonellae are some of the most commonly isolated bacteria from bacteraemia in susceptible infants or immunocompromised adults globally (Deen et al., 2012; Feasey et al., 2012). Of the non-typhoidal salmonellae, some serovars such as *S.* Cholerasuis and *S.* Dublin are more invasive and cause higher mortality compared to others (Jones et al., 2008). Different non-typhoidal *S. enterica* serovars also cause significant morbidity and mortality in various livestock species, representing a threat to animal health and welfare as well as human health and food security.

1.1.1 - Mechanism of infection

S. enterica transmission and infection occurs largely via the faecal oral route, whereby food or water contaminated with infected waste is consumed and the bacteria gain entry to the gastrointestinal tract (Nielsen, 2013a). The gastrointestinal tract and intestinal epithelial cells act as the "first line of defence" from invading enteric pathogens whilst preventing inappropriate immune responses to non-pathogenic commensal microorganisms (Broz, Ohlson, and Monack, 2012). However, several strategies have evolved in *Salmonellae* which allow the bacteria to breach the intestinal epithelium and gain access to the circulatory and lymphatic systems. These strategies include invasion of the microfold "M" cells of Peyers patches, aggregated lymphoid tissue involved in immune surveillance of the intestine (Jones, Ghori, and Falkow, 1994; Monack et al., 2000). *Salmonallae* can also directly invade and destroy enterocytes, possibly providing opportunity for the invasion of the interstitial space and thereafter the lymphatic system (Frost, Bland, and Wallis, 1997; Pullinger et al., 2007). Another potential mechanism of host intestinal infection is *Salmonella* invasion of resident phagocytes and subsequent dissemination (Vazquez-Terres et al., 1999). These mechanisms of invasion appear to be host species specific as demonstrated by the differences in host tissue invasion observed in mouse, rabbit and guinea pig models (Giannella et al., 1973; Jones, Ghori, and Falkow, 1994; Takeuchi,

1967). Dissemination is thought to occur in the intracellular niche of phagocytes, either in the lymphatic system or circulatory system, although studies have shown contribution of free-living bacteria in the lymph and bacteraemia can accompany infection (Dias et al., 2009; Nielsen, 2013a; Pullinger et al., 2007). Not all *Salmonella* infections result in systemic dissemination and bacteraemia however, with many causing self-limiting diarrhoea, vomiting and pyrexia (Chen et al., 2013). This is partly due to differences in host susceptibility which is influenced by age and immune status, but is also impacted by bacterial virulence (Stanaway et al., 2019).

1.1.2 - *Salmonella* virulence

The virulence of any bacterial species is related to their ability to infect and colonise a host (Waldner et al., 2012). Virulence can be divided generally into different functional characteristics which includes host cell adherence and invasion, intracellular survival and replication, motility and the ability to out-compete other bacteria during colonisation.

1.1.2.1 - Host cell adherence and invasion

Some of the best characterised virulence mechanisms in *Salmonellae* are the Type Three Secretion Systems (T3SSs) chromosomally encoded predominantly on *Salmonella* Pathogenicity Islands (SPIs) 1 and 2. T3SS are needle-like structures, evolutionarily related and structurally similar to the flagellar basal body, which span the inner and outer membrane of the bacteria and protrude into host cells (Abby and Rocha, 2012; Kubori et al., 1998). Inside the bacterium, C-rings pass through the inner and outer bacterial membrane and surround an inner rod, export apparatus and sorting platform which controls the timing of effector secretion (Lara-Tejero et al., 2011) (Figure 1.1).

The SPI-1 T3SS is essential for *Salmonella* virulence in various species and tissues. SPI-1 expression *in vitro* is regulated by *hilA,* expression of which is induced by changes in environmental and physiological parameters like alterations in pH, osmolarity and

oxygen availability (Bajaj et al., 1996; Lostroh and Lee, 2001). In the SPI-1 T3SS, PrgI proteins form the helical structures that make up the needle with an inner diameter of ~20 A° through which effector proteins are injected (Hu et al., 2017; Roblin et al., 2015). This needle section of the "injectisome" requires a channel or pore to be formed by proteins SipB, SipC and SipD in the host cell membrane (Ochman et al., 1996; Zhang et al., 2002). PrgH and PrgK are also involved in forming the channels through which the needle protrudes around 65nm into the cytoplasm of the host cell (Hu et al., 2017; Makino et al., 2016). Secreted effector proteins like SopE and SopB trigger host cell actin polymerisation by activating Cdc42 and Rac-1 intracellularly (McGhie, Hayward, and Koronakis, 2004). The remodelling of the host cell cytoskeleton creates characteristic "membrane ruffles" observed in *Salmonella*-infected cells (Darwin and Miller, 1999). The bacterium is then engulfed inside the *Salmonella* containing vacuole (SCV) inside the host cell, a process similar to but distinct from phagocytosis and endocytosis (Cain, Hayward, and Koronakis, 2008; Cossart, 2004).

Other bacterial species are destroyed due to fusion of the endosome with bactericidal components inside cells, but *Salmonella* prevent lysosomal fusion to promote intracellular survival and replication (Buchmeiert and Heffront, 1991). The second T3SS encoded on SPI-2 is activated in the intracellular environment and produce SifA (*Salmonella* induced filament), SopD2 and SseJ which alter the process of endosomal maturation and maintain the SCV, generating a unique intracellular niche (Beuzón et al., 2000; da Silva et al., 2012; D'Costa et al., 2015; Jiang et al., 2004; Knuff and Finlay, 2017; McGourty et al., 2012; Ruiz-Albert et al., 2002). *Salmonella* induced filaments are associated with bacterial localisation within host cells and have been shown to aid in acquisition of endocytosed nutrients which aid in bacterial metabolism and replication (Liss et al., 2017). SPI-2 is important in the survival and replication in various cell types, including macrophages and epithelial cells, and its inactivation is associated with reduced or attenuated virulence in animal models

(Figueira et al., 2013; Jones et al., 2001; Salcedo and Holden, 2003). It is not clear how precisely *Salmonellae* are disseminated systemically, but the ability of the bacteria to enter and replicate within an almost immune-privileged niche suggests that intracellular dissemination inside immune cells is a possibility.

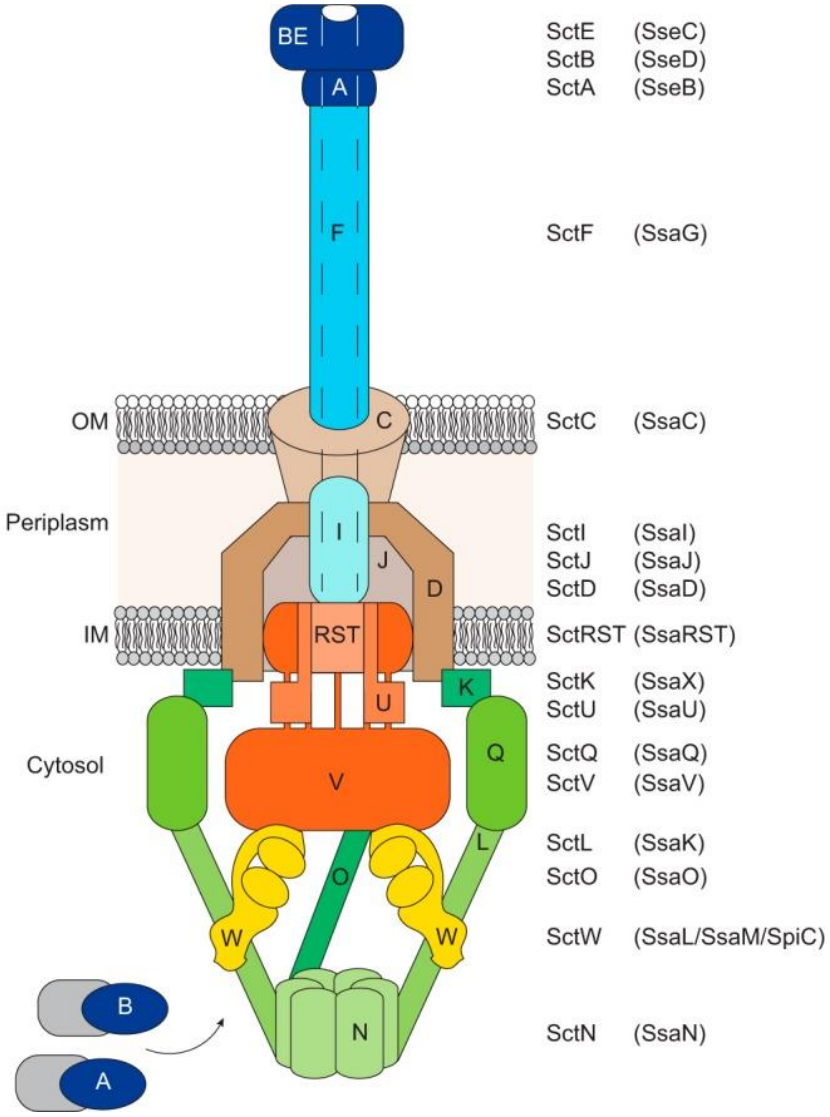


Figure 1.1 – Schematic of the *Salmonella* Pathogenicity Island (SPI) 2 Type Three Secretion System (T3SS). From Yu et al., 2018.

An essential step in host cell invasion involves adherence to the target cell. Fimbriae are involved with host cell adherence as well as biofilm formation and persistence in macrophages and can regulate the expression of the SPI-1 invasion phenotype via *hil* modulation (Baxter and Jones, 2005; Boddicker et al., 2002; López-Garrido and Casadesús, 2012; Sabbagh et al., 2012). Fimbriae are non-flagellar

filamentous structures expressed on the outer membranes of bacteria (Rehman et al., 2019; Weening et al., 2005). Different fimbriae are involved with adhesion to different types of cells, potentially indicating a role in host cell recognition for *Salmonellae* (Bäumler et al., 1996; Bäumler, Tsolis, and Heffron, 1996; Humphries et al., 2001). Fimbriae are categorised into Type 4 (IV-B), nucleated precipitation and chaperone usher, which is further categorised into α, β, γ (of which there are four types), κ, π and σ (Rehman et al., 2019). The evolution of different *Salmonella* serovars has been accompanied by the gradual acquisition and loss of non-advantageous fimbrial operons, again indicating the potential for both host tissue and host species recognition (Yue et al., 2012). As well as promoting bacterial adhesion, fimbriae can be potent stimulators of the host immune system, whereby initiating intestinal inflammation can be associated with increased virulence (Kuzminska-Bajor, Grzymajlo, and Ugorski, 2015).

1.1.2.2 - Modulation of host immunity to promote survival

As well as allowing for host-cell invasion by initiating membrane-ruffling and bacterial endocytosis, SPI-1 is also involved in activating host intestinal immunity which promotes bacterial survival. Various SPI-1 secreted effector proteins including SopA, SopB, SopD, SopE2 and SipA activate host immunity via the NF-κβ pathway, stimulating production of neutrophil chemoattractant IL-8 (McCormick et al., 1995; Zhang et al., 2002). The infiltrating neutrophils and immune activation destabilise the tight junctions between intestinal epithelial cells, providing a mechanism for bacterial infiltration of the interstitial space (Pullinger et al., 2007). Therefore, upregulation of the immune system in response to the presence of *Salmonella* in the intestine can convey an advantage to the bacteria and allow for intestinal infiltration. Conversely, expression of SPI-1 gene *avrA* is associated with inhibition of NF-κβ signalling, thereby modulating the immune response (Collier-Hyams et al., 2002; Wu, Jones, and Neish, 2012).

1.1.2.3 - Competition with commensal bacteria during colonisation

In addition to evading detection and destruction by host defences, *Salmonella* must compete with the normal gut flora in the early stages of colonisation (Dostal et al., 2014).

During intestinal inflammation and the initial stages of host invasion, mucosal cells and neutrophils express lipocalin-2 (LCN2) as a strategy to reduce available iron to limit bacteria growth (Goetz et al., 2002; Singh et al., 2016). However, *Salmonellae* have evolved strategies to mitigate against the impact of LCN2 via the production of siderophores like enterobactin and salmochelins via expression of *ent* and *iro* operons (Hantke et al., 2003; Raymond, Dertz, and Kim, 2003). These iron-chelating molecules allow for *Salmonella* growth and aid in intestinal colonisation in a niche limited in iron (Raffatellu et al., 2009). Furthermore, the iron-limited environment produced as a result of intestinal inflammation can result in the death of commensal bacteria, allowing *Salmonellae* to out-compete in the inflamed intestine.

As well as T3SS, *Salmonella* genomes encode for Type Six Secretion Systems (T6SS) on SPI-6 and infrequently SPI-19, present in a limited number of *Salmonella* serovars (Blondel et al., 2009; Langridge et al., 2015; Mohammed and Cormican, 2016; Mohammed et al., 2017). Researchers have compared T6SSs to crossbows, whereby contractile elements of the structure encoded by *tssB* and *tssC* launch to penetrate nearby competing bacteria with *hcp* encoded tubules and deliver antimicrobial effectors (Sana et al., 2016; Sana, Lugo, and Monack, 2017; Silverman et al., 2012). Antimicrobial effector proteins like Tae4 are injected and induce bacterial lysis, allowing the *Salmonella* to out-compete other bacteria by directly killing their competition (Benz, Reinstein, and Meinhart, 2013).

1.1.2.4 - Motility

Motility is highly important in terms of bacterial virulence as bacteria can move towards nutrient resources or towards advantageous

niches, or away from disadvantageous environments. *Salmonella* Typhimurium often expresses between 6 and 10 flagella, composed of proteins encoded by around 60 different genes (Bonifield and Hughes, 2003; Frye et al., 2006). Flagella are structurally similar to T3SSs and comprise a basal body (including inner and outer membrane C-rings, as in T3SSs), a hook and flagellar filament (Figure 1.2). Rotation of the flagellar filament driven by protonmotive force across the bacterial membrane provides bacterial motility in a directional and chemotactic manner (Berg, 2003). Along with other outer membrane surface structures like fimbriae, expression of flagella is also associated with biofilm formation, conveying resistance to antibiotics, disinfectants and components of host immunity, thereby increasing bacterial virulence (Beshiru, Igbinosa, and Igbinosa, 2018; Høiby et al., 2010; Wang et al., 2020). Like many outer-membrane structures, flagellum are highly immunogenic and therefore, some serovars have adapted to repress flagellar expression during invasion to subvert host immune responses (Spöring et al., 2018; Yim et al., 2014). Interestingly, some serovars do not possess flagellar motility but are virulent in their host niches (Cheraghchi et al., 2014). In *S.* Gallinarum, the *fliC* gene has undergone heavy pseudogenisation over time, a result of the process of host adaptation (Li et al., 1993).

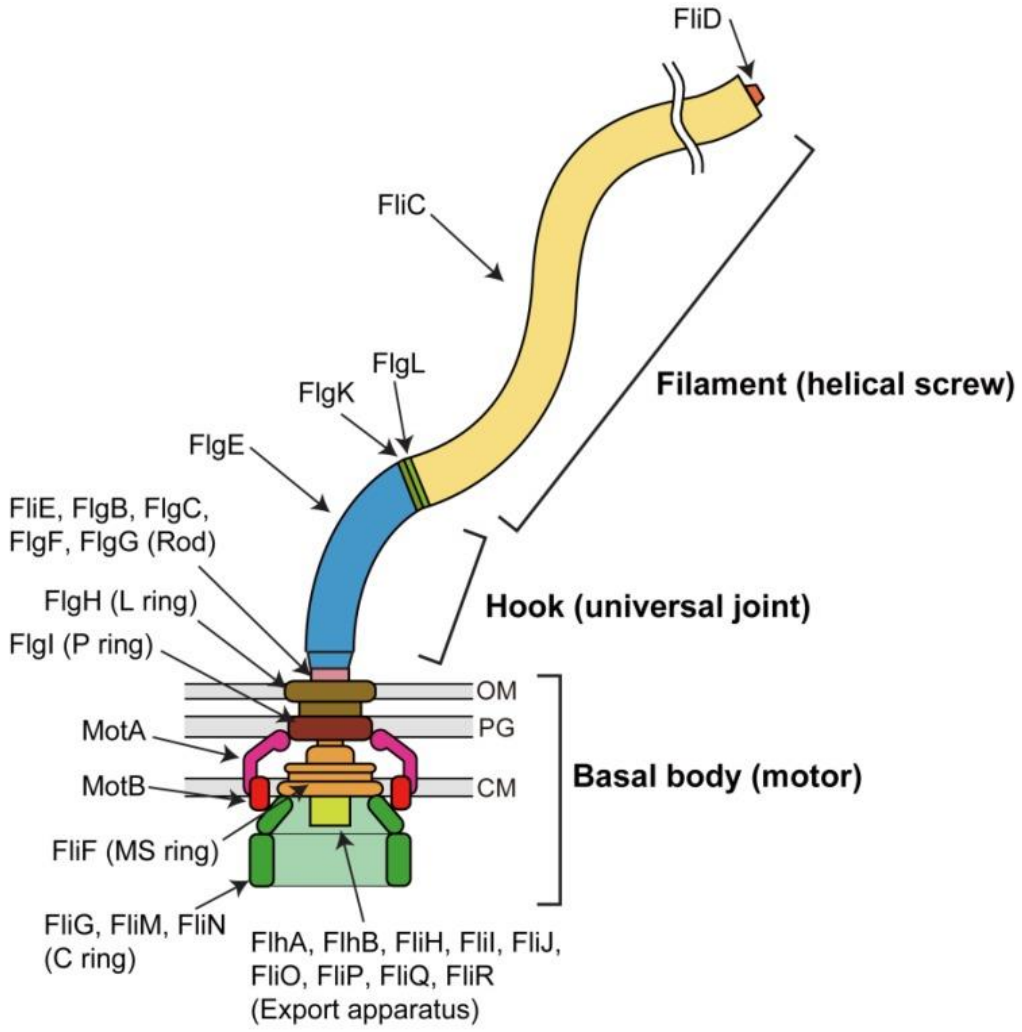


Figure 1.2 – Schematic of the flagellar motor structure in Enterobacteriaceae. From Morimoto and Minamino, 2014**.**

1.1.3 - Host adaptation of *Salmonella enterica* serovars

The process of host adaptation involves the acquisition of genes conveying advantages in particular species niches, followed by the gradual loss or pseudogenisation of genes which are no longer required for bacterial virulence or fitness in that particular niche. The evolution of *Salmonella spp.* towards pathogenicity is thought to have occurred largely through horizontal gene transfer. This is exemplified by the fact that SPI-2 has a different G-C content compared to the rest of the genome, indicating that it was likely to have been acquired from another pathogenic species (Shea et al., 1996). The acquisition of virulence factors conveying these advantages can be carried on mobile genetic elements like plasmids and phage. Acquisition of

virulence via the transfer of plasmids and other mobile genetic elements is often referred to as a "quantum leap" in the literature because of how quickly this can impact bacterial virulence (Mirold et al., 2001). In many *Salmonella* the plasmid-encoded *spv* operon impacts bacterial virulence by modulating the host immune response (*spvC* and *spvD*) and actin polymerisation (*spvB*) (Lesnick et al., 2001; Hongtao Li et al., 2007; Rolhion et al., 2016). Gifsy-2 phage encode SodC1 which conveys virulence in murine macrophages (Figueroa-Bossi and Bossi, 1999). Antimicrobial resistance genes are often acquired via horizontal gene transfer of plasmids, including the IncA/C type plasmids which can confer resistance to multiple antibiotics (Hoffmann et al., 2017). The process of host adaptation also includes the loss of genes which are not essential for virulence or survival in a particular niche. Phylogenetically related serovars Gallinarum, Enteritidis and Dublin all harbour SPI-6 and SPI-19, yet pseudogenisation of SPI-19 in *S.* Enteritidis indicates that this island is not essential for *S.* Enteritidis pathogenicity (Blondel et al., 2010). In contrast, SPI-19 has been shown to be essential in *S.* Gallinarum infection of chicken macrophages (Blondel et al., 2013). However, as *S.* Gallinarum and *S.* Dublin are host restricted and adapted serovars respectively, and *S.* Enteritidis is a host generalist, the expected higher number of pseudogenes is observed in *S.* Gallinarum and *S.* Dublin compared to *S.* Enteritidis (Betancor et al., 2012; Langridge et al., 2015).

1.2 - *Salmonella enterica* subspecies *enterica* serovar Dublin

Salmonella enterica subspecies *enterica* serovar Dublin (*S.* Dublin) is a host adapted *Salmonella* serovar and the most commonly isolated *Salmonella* serovar implicated in disease in cattle in the UK (APHA, 2021a; Veterinary Medicines Directorate, 2019). Unlike host-restricted *Salmonella* serovars like *S.* Paratyphi in humans and *S.* Gallinarum in fowl, *S.* Dublin is known to cause serious disease in a number of different hosts (Waldner et al., 2012). *Salmonellae*

including *S.* Dublin are notifiable diseases in the UK, partially due to the fact that it is zoonotic. Whilst uncommon, there are confirmed cases of human salmonellosis caused by *S*. Dublin which have usually occur sporadically and in isolation (Mohammed et al., 2017; Fang and Fierer, 1991). Many of these cases have been traced to the consumption of unpasteurised milk products, where an asymptomatic carrier excretes the bacteria in milk (Avenue, 1992). Contaminated beef products can also act as a reservoir for *S.* Dublin as bacteria often resides in lymph nodes which are not removed during normal carcass processing (Vohra et al., 2018). Infections with *S*. Dublin are known to be more severe and have a higher mortality rate than infections with other *Salmonella* serovars (Helms et al., 2003; Ersbøll and Nielsen, 2008). Human disease with Dublin results in higher hospitalisation rate and mortality of 67% and 3% respectively reported in one study (Jones et al., 2008). *S.* Dublin is therefore a concern for both animal health and welfare, and human health and food security. *S.* Dublin is globally recognised as an issue impacting animal and human health.

1.2.1 - *Salmonella* Dublin infection of cattle

Infection with *S.* Dublin can occur in animals of any age and cause a range of clinical outcomes. *S.* Dublin infection, like infection with other serovars, occurs via the faecal oral route (Holschbach and Peek, 2018). Transmission can occur via contaminated pasture, contact with other infected animals, consumption of contaminated feed stuffs, or through environmental contamination from other infected sites due to poor biosecurity (Davison et al., 2006; Davison et al., 2005; Fenton et al., 2009). It is possible that direct uptake of the bacterium into the tonsils and suprapharyngeal lymph nodes could contribute to the establishment of infection, though little research has been conducted on this mechanism of colonisation (Nazer and Osborne, 1977). Intestinal colonisation and invasion of the lymphatic and circulatory systems has been demonstrated in experimentally infected cattle, where the bacterium invades the intestinal epithelium (Pullinger et al., 2007). Studies into the impact of *S.* Dublin on the

intestinal epithelium note the considerable histological changes observed with *S.* Dublin (Bolton et al., 1999; Landsverk et al., 1990). Isolation of *S.* Dublin from jugular vein blood and efferent lymph (away from the gastrointestinal tract) in cell-free niches demonstrates two potential methods of dissemination in whole animal models (Pullinger et al., 2007). *S.* Dublin has been isolated from a variety of systemic locations, including various lymph nodes, spleen, kidney, liver, lungs and reproductive tract, including the placentome and fetus in pregnant animals (Figure 1.3, summarised in Table 9.1). Isolation of the bacterium from the liver and kidneys can be indicative of bacteraemia and is indicative of systemic translocation (Carrique-Mas et al., 2010). The presence of the bacterium in lymph nodes has been suggested to elude to previous infection, as animals previously infected can harbour *S.* Dublin in lymph nodes and are then subject to recurrent infection (Nielsen et al., 2004; Webber et al., 2009). Recurrent infection is thought to occur due to environmental stressors or changes in the animals immune status and can initiate shedding of the bacteria in milk, urine and faeces (Nielsen, 2013a). The shedding of bacteria in urine and faeces, combined with the propensity of *S.* Dublin to persist for long periods of time in the environment, means that it is exceptionally difficult to eradicate on farm (Kirchner et al., 2012).

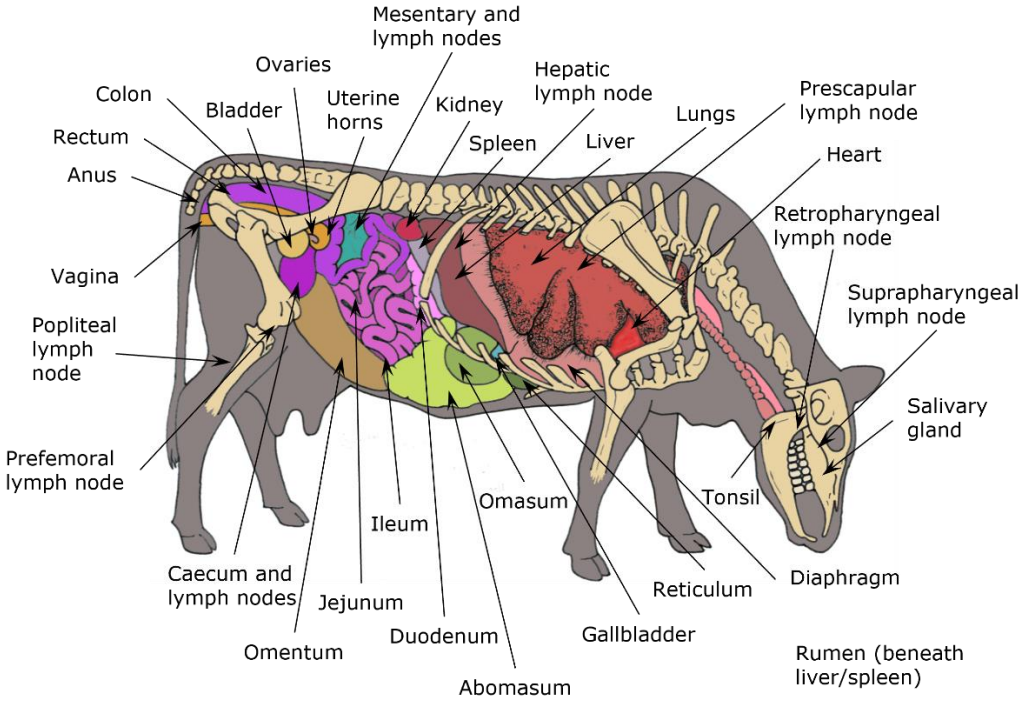


Figure 1.3 – Anatomical structures of the cow, referencing the different tissues from which *S.* Dublin has been isolated in previous studies. Details of these studies are summarised in Appendix Table 9.1. Diagram adapted from Ball and Peters, 2004a and Budras et al., 2011.

1.2.2 - Control of *S.* Dublin on farm

Due to the impact of any disease on the health and welfare of livestock and the consequences of this on productivity and therefore profitability, farmers should be encouraged to do all they can to control infectious agents on farm. This is likely to be achieved by using a combination of approaches, from treatment of infected animals to preventing disease spread by maintaining good biosecurity and utilising vaccination.

1.2.2.1 - Biosecurity to prevent *S.* Dublin incursion on farm

For farms without a prior history of *Salmonella*, maintaining a closed herd is likely the most effective prevention of *S.* Dublin (Davison et al., 2006). Breeding replacement stock on-site reduces the likelihood of introducing an already-infected animal, and using artificial insemination also reduces the risk of introducing an infection into a herd as only healthy animals can be used for semen collection. If replacement animals are brought into a herd, animals should be

quarantined whilst it is ascertained as to whether the animal is healthy, particularly as sub-clinical disease can allow for shedding of the bacteria. Avoidance of cattle markets and similar events is also recommended to avoid contact with sub-clinically infected animals (Davison et al., 2006). Recommendations for management include proper training of personnel and appropriate record keeping, particularly for recognising reproductive failures and abortion (Hovingh, 2002). Adequate colostrum should be given to new-born calves in order to provide the immunoglobulins essential for survival and immunise calves against diseases present in the immediate environment (Nielsen et al., 2013). Separation of younger and older animals is key to avoiding horizontal transmission of diseases, as older animals may already be immune to some diseases and act as vectors. Younger animals are put at risk of disease acquisition when mixed with older animals, so proper management and separation of these animals from the rest of the herd aids in preventing transmission to somewhat immunocompromised youngstock (Carrique-Mas et al., 2010).

Once *S*. Dublin has been introduced into a herd, its ability to survive in the environment for long periods of time makes it is very difficult to eradicate (Nielsen and Dohoo, 2012). Viable bacteria have been found in soil, manure and slurry months after excretion (Waldner et al., 2012). Therefore, proper pasture management is of vital importance, including ensuring that potentially contaminated manure is not used to fertilise fields designated for livestock rotations. Wildlife reservoirs have been reported for many infectious diseases, including *S.* Dublin and whilst unlikely, the possibility for mammals like badgers to spread the disease has been suggested (Haw, 1977; Wilson et al., 2003). Therefore, proper boundary maintenance and pest control could also be implicated in prevention of *S.* Dublin infection.

1.2.2.2 - Vaccination against *S.* Dublin

Bovivac S, a vaccination against *S.* Dublin and *S.* Typhimurium produced by MSD Animal Health is currently available in the UK

(Henderson and Mason, 2017). This vaccine contains formalin-killed cells of *S.* Dublin strain S342/70 and Typhimurium strain S341/70 at $1x10^9$ cells per millilitre, as well as aluminium hydroxide gel adjuvant at 200mg/ml (NOAH Compendium, 2017). A survey-based study into the uptake and use of cattle vaccines in the UK found that Bovivac S was used by only around 9% of respondents (Cresswell et al., 2014). This is likely to be insufficient to help eradicate the disease but may allow the prevention of disease occurrence within a single herd, provided repeated vaccination occurs and correct biosecurity measures are followed (Hovingh, 2002). A vaccine commercially available in the US targeting the siderophores produced by *S.* Newport has been shown to be poor in controlling *S.* Dublin and demonstrates that vaccines likely need to be serovar specific (Kent et al., 2021).

1.2.2.3 - Antimicrobial Resistance in *Salmonellae:*

Treatment of *S.* Dublin infection largely involves supporting the infected animal with fluid therapy (Hovingh, 2002). Whilst it is currently not possible to ascertain the general use of antibiotics in the treatment of *S.* Dublin in the UK, antimicrobial resistance is an important facet of infection which must be mentioned.

The term "antibiotic" was first used by Selman Waksman in 1941 to describe the compounds produced by microorganisms which prevented the growth of (bacteriostatic) or killed (bactericidal) bacteria (Clardy, Fischbach, and Currie, 2009). Antibiotic mechanisms are broadly categorised into inhibition of protein, DNA, RNA and cell wall synthesis. Intrinsic resistance is conveyed when bacteria possess a characteristic which prevents antimicrobial action without the need to acquire it from other species of bacteria, exemplified by the intrinsic resistance of gram-negative bacteria to the action of vancomycin due to its inability to penetrate the cell wall (Fernandes et al., 2017). Aside from intrinsic resistance, bacteria have evolved a variety of genes conveying resistance to antimicrobials, driven in part by evolution to escape natural sources

of antimicrobials. However, the use (and over or mis-use) of antimicrobials in human and animal medicine has also provided selection pressure to drive the development and acquisition of antimicrobial resistance genes across a range of bacterial species.

Antimicrobial resistance is rarely seen in the UK in *S.* Dublin isolates, but resistance is observed on occasion to ampicillin, chloramphenicol, nalidixic acid, neomycin, streptomycin, sulphonamides and tetracycline (Veterinary Medicines Directorate, 2020). Ampicillin resistance can be conveyed by the bla_{TEM} gene which inactivates β-lactam antibiotics by hydrolysing the β-lactam ring (Livermore, 1995; Srednik et al., 2021). Efflux pumps prevent accumulation of antibiotics inside the bacterium and therefore prevent the action of antibiotics like chloramphenicol (for example, AMR gene *floR*) and tetracycline (*tet* genes) (Braibant et al., 2005; Kumar and Varela, 2012). Multi-drug IncA/C plasmids can harbour many of these resistance genes and more, and have been identified in *S.* Dublin isolates of cattle origin previously (Hoffmann et al., 2017; Paudyal et al., 2019).

Antimicrobial resistant *S.* Dublin poses a threat to both human and animal health and welfare, as prolonged and potentially incurable illness can ensue from infection with multi-drug resistant isolates. Moreover, in humans, infection with multidrug resistant nontyphoidal *Salmonella* appears to result in more clinical symptoms and disease outcomes than those without resistance to antibiotics (Parisi et al., 2018).

1.2.3 - Clinical signs of *S.* Dublin in cattle

Clinical signs of *S.* Dublin infection in cattle vary considerably, largely due to the age of the animal, infectious dose and any conditions meaning that the animal is in some way immunocompromised (summarised in Table 1.1). The duration of the infection and shedding of the bacteria in faeces, milk and urine are also dependent upon these factors. Some infections can occur without clinical signs at all, like with passive carriers where the bacterium resides in the gut and

bacteria can be shed in faeces until the infection is cleared, or animals with subclinical infection of lymph nodes which may be reactivated due to stress. Other infections result in diarrhoea and pyrexia and loss of appetite. Respiratory distress and pneumonia is also reported in calves infected with *S.* Dublin (Pecoraro, Thompson, and Duhamel, 2017). For pregnant animals, infection with *S.* Dublin can lead to abortion, with no other clinical signs preceding the abortion event (Hinton, 1977; Vaessen et al., 1998).

Table 1.1 - Clinical characteristics, duration and infectiousness of different infection stages of *S*. Dublin upon oral uptake in susceptible cattle. Adapted from (L. R. Nielsen, 2013b)**.**

Infection stages and their characteristics	Duration	Shedding of bacteria
Acute infections		
Preacute Death following bacteraemia and endotoxic shock, without other clinical signs. Most common in naïve herds.	1-2 days	Animals often die before bacterial excretion begins.
Acute Local enteric infection or systemic infection with transient bacteraemia. Additional clinical signs in calves and adults sometimes seen (eg: loss of appetite, hyperthermia, diarrhoea).	1-3 weeks, may extend to 5-9 weeks	May shed large amounts (from 1 to 10^8 CFU/g) in faeces, urine, vaginal discharge and milk.
Persistent infections		
Chronic infection Follows acute infection usually in calves older than 6-8 weeks. Clinical signs include bloody/loose stool, shedding of intestinal casts, slightly elevated temperature, arthritis and ischaemic necrosis of the skin on ears, tail or distal limbs.	Months	The animal may or may not be shedding bacteria.
Passive carrier Subclinical. Passive carrier of bacteria in gut lumen, no invasion of the intestinal epithelium.	Weeks to months	Shedding periodically in faeces until removed from source.
Latent carrier Subclinical, bacteria in lymphoid tissues. Can become reactivated through stress.	Months to years	Shedding low amounts only when reactivated.
Active carrier Subclinical. Active carriers (by some referred to as supershedders). May carry the bacteria in both the lumen of the gut, gut-associated tissues, lymphoid system and internal organs.	Months	Intermittent or continuous shedding at similar levels as acutely infected.

1.2.4 - Incidence of *S.* Dublin abortions in cattle in the UK

S. Dublin is one of the most common causes of bovine abortion in the UK, along with *Neospora caninum, Bacillus licheniformis, Trueperella pyogenes* and congenital Bovine Viral Diarrhoea virus (BVD) (Figure 1.4 and Figure 1.5) (APHA, 2021a). *S.* Dublin was the most common cause of bovine abortion in 2019, 2020 and 2021 despite the number of abortions generally in the UK declining year on year (Figure 1.5). The incidence of *S.* Dublin appears to be cyclical, with most abortions attributed to this pathogen occurring later in the year (APHA, 2019b). This may coincide with herds being brought into sheds for winter, increasing the likelihood of infections spreading from one animal to another due to increased animal-animal contact (Davison et al., 2006; Vaessen et al., 1998).

It is highly likely that the prevalence of abortions due to *S.* Dublin are greatly underreported due to a variety of factors (Nielsen et al., 2004; Weston et al., 2012). The process of pregnancy diagnosis in cattle is in itself challenging, with animals being culled due to infertility, yet being found to be pregnant following slaughter (Noakes, Parkinson, and England, 2009). An abortion rate of less than 5% is routinely tolerated in the UK, and whilst UK law states that abortions must be investigated, it is possible that this legislation is not always followed, as has been shown in France (Bronner et al., 2014; Wheelhouse and Dagleish, 2014). Furthermore, investigation of bovine abortion in May of 2022 costs £90 per test which may be off-putting for farmers (APHA, 2022). Additionally, sampling of the tissues required to report a bovine abortion may spend a long time in transit, as there are a limited number of diagnostic laboratories in the UK. This could mean that diagnosis is not possible, contributing to the number of undiagnosed cases of bovine abortion. Between the years 2002 to 2016 in the UK, an average of only 10.4% of all abortions were diagnosed (13071 out of 125984), with the vast majority defined as Brucellosis negative and otherwise inconclusive (APHA, 2016).

1.2.5 - Reporting of bovine abortion in the UK

UK legislation enforces reporting of all abortions in cattle to the Animal and Plant Health Agency (APHA) for further investigation, primarily to ascertain whether Brucellosis is a likely cause (Animal Health Act (1981), 2015). Cattle owners must notify the Veterinary Head of Field Delivery within 24h of the abortion occurring (DEFRA, 2018). Abortion enquiries are pursued in animals which have not previously calved, the animal was born outside of Great Britain or Northern Ireland or if there have been other abortions or premature calvings in the herd in the previous month (DEFRA, 2018). APHA administrative staff then use a decision tree to determine if an abortion enquiry is required if the previously stated circumstances are not fulfilled (Appendix Figure 9.1). An outcome of this process can be that investigation into the cause of abortion is not required, in which case placental and fetal tissues can be disposed of (DEFRA, 2018). When investigations are required, the animal must be in isolation and the fetus and afterbirth are sampled. Veterinary practitioners take a full clinical history of the animal including the type of herd, age of the dam and previous abortion history, biosecurity and vaccination status. The practitioner then takes samples of the placentome and fetal stomach contents, fetal brain, fetal spleen and thymus, and blood from the dam (APHA, 2021b). Fetal stomach contents are used for culture of causative bacteria, where *Salmonellae* would be identified (APHA, 2021b).

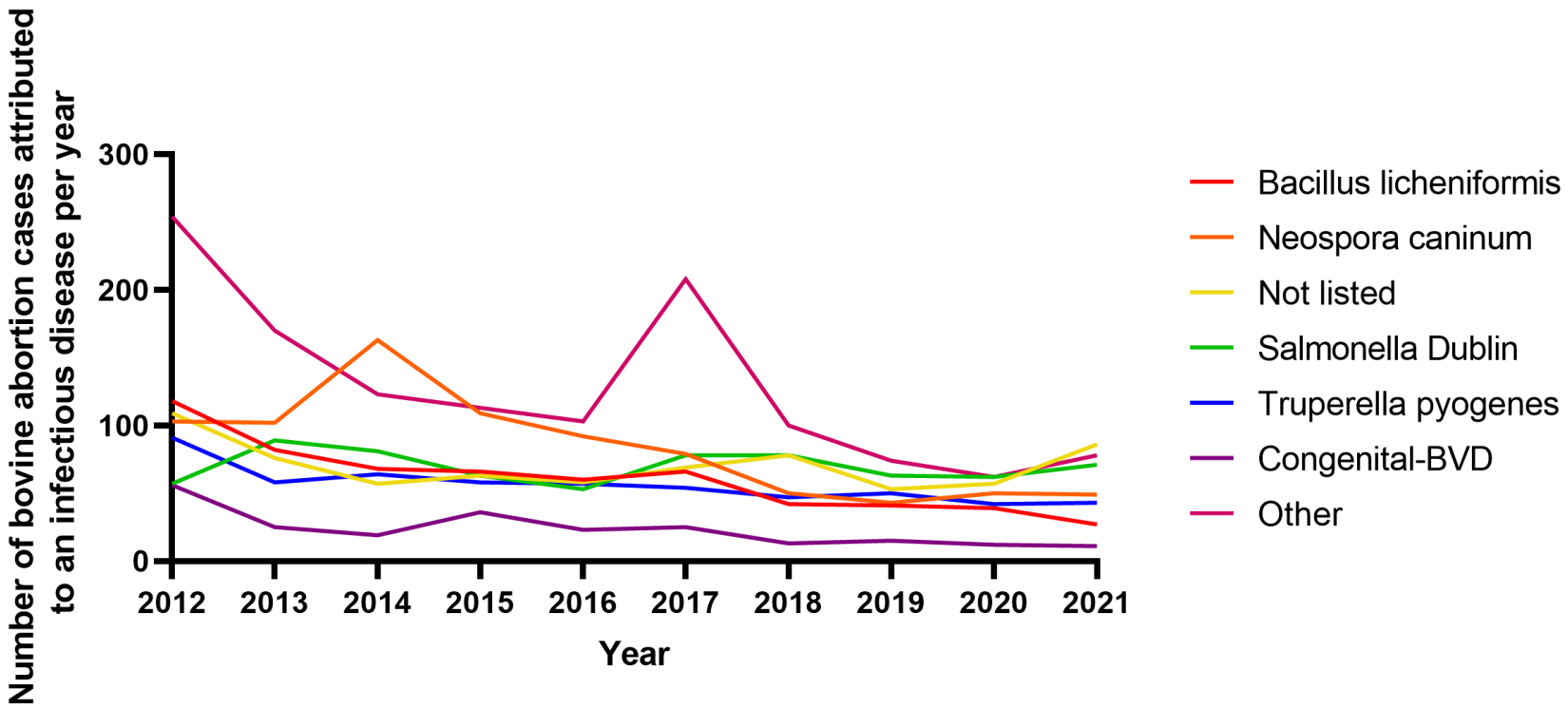


Figure 1.4 – Number of cases of bovine abortion attributed to infectious diseases each year in the UK from 2012 to 2021. Data from the Cattle Disease Surveillance Dashboard (APHA, 2021a)**.** "Not listed" refers to when a diagnosis has been reached but there is not a specific Veterinary Investigation Diagnosis Analysis (VIDA) code for the diagnosis. "Other" refers to the total number of animals diagnosed with either *Coxiella burnetti, Campylobacter, E. coli, Leptospira, Listeria, Salmonella* Typhimurium, *Salmonella* (not otherwise specified), Schmallenberg virus, "fungi", "Enzootic" or "IBR/IPV", which were added together as there were too few of each to specify in this figure. Data from 2020 is limited because of the impact of the COVID-19 pandemic on the collection of samples and diagnosis of causative agent.

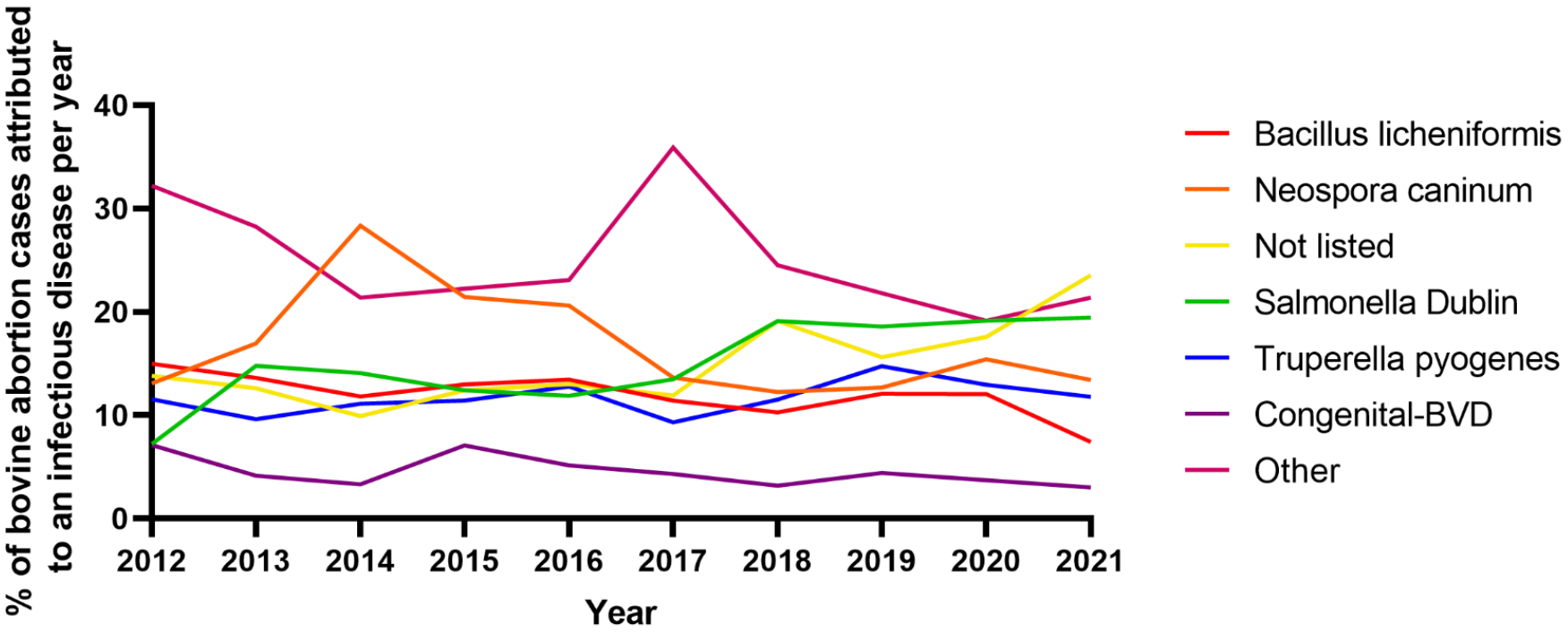


Figure 1.5 – Percentage of cases of bovine abortion attributed to infectious diseases each year in the UK from 2012 to 2021. Data from the Cattle Disease Surveillance Dashboard (APHA, 2021a)**.** "Not listed" refers to when a diagnosis has been reached but there is not a specific Veterinary Investigation Diagnosis Analysis (VIDA) code for the diagnosis. "Other" refers to the total number of animals diagnosed with either *Coxiella burnetti, Campylobacter, E. coli, Leptospira, Listeria, Salmonella* Typhimurium, *Salmonella* (not otherwise specified), Schmallenberg virus, "fungi", "Enzootic" or "IBR/IPV", which were added together as there were too few of each to specify in this figure. Data from 2020 is limited because of the impact of the COVID-19 pandemic on the collection of samples and diagnosis of causative agent.

1.3 - Reproduction in Cattle

Beef and dairy produce for human consumption is heavily reliant on the efficiency of reproduction in cattle. Dairy cows must fall pregnant and calf in order to maintain lactation, whilst beef cows produce animals which will be reared and slaughtered for meat. Due to the length of bovine gestation, a cow will produce one calf per year at best, so it is crucial that the animal will become pregnant and successfully produce offspring (Ball and Peters, 2004b). Therefore, understanding and prevention of abortion is of the utmost importance for milk and beef production. Additionally, infection and resultant abortion represents a welfare issue, as animals should be free from disease distress under the five freedoms (Brambell et al., 1965).

1.3.1 - Anatomy of the bovine female reproductive tract and pregnancy

Ungulates like cows, sheep and deer share similarities in the structure of their reproductive tracts. Like in most mammals, the vulva forms the exterior of the reproductive tract with the vagina extending anteriorly (Ball and Peters, 2004a). The cervix, a thick ring of fibrous tissue, remains closed until the dam gives birth where it relaxes under the influence of various hormones (Bondurant, 1999); (Ball and Peters, 2004a) (Figure 1.6, A). The ungulate uterus is distinct from that in other mammals, as it comprises of a uterine body and two uterine horns, also described as a bicorunate uterus (Senger, 2012). The uterine horns account for the majority of the uterine space, whilst the uterine body in cows is comparatively small (Figure 1.6, B). The uterine wall consists of the endometrium, myometrium and serosa (Ball and Peters, 2004a). The endometrium, or inner lining of the uterus, is the main site of placental attachment during pregnancy. The myometrium, a muscle-rich tissue, aids in expulsion of the foetus during parturition, and the serosa surrounds this whole structure (Ball and Peters, 2004a). The uterus is lined with caruncles which aid in placental attachment during pregnancy (Figure 1.6, B) (King,

Atkinson, and Robertson, 1980). The average length of gestation in the cow is around 280 to 285 days (Ball and Peters, 2004b).

1.3.2 - Bovine placentation

Bovine placentation is minimally invasive, where maternal carunclular tissues interdigitate with fetal cotyledonary tissues. The placentome is synepitheliochorial, meaning that maternal and fetal blood are separated by several distinct cell layers, namely the namely the maternal epithelium, fetomaternal syncytium and fetal trophoblast cells (Peter, 2013; Robbins and Bakardjiev, 2012). The structure of the bovine placentome is vastly different to the placentas of humans or mice in that there are more cell layers between the fetal and maternal blood supplies (Robbins and Bakardjiev, 2012). The maternal caruncular tissues of the bovine placentome are convex with the fetal cotyledonary tissues overlaying the maternal tissues, whereas the maternal caruncular tissues in the ovine placentome are concave, demonstrating variation in placentation even between ungulates. Following fertilisation and differentiation into the blastocyst, the trophoblast cell layer along with the somatic mesoderm forms the chorioallantois from around 20 days post conception (Schlafer, Fisher, and Davies, 2000). During the development of the chorioallantois, the caruncular tissues already present in the uterus begin to form villi around 28 days post conception (Wooding and Burton, 2008). The mutual production of growth factors from these tissues aid in their extension towards one another and the eventual formation of the placentome structure (Figure 1.6, C). Trophoblast cotyledonary tissues interdigitate with the maternal caruncles to produce the "popper-like" placentome (Bridger, Menge, et al., 2007; Wooding and Burton, 2008). The placentome is a principle site of nutrient exchange, with a rich vasculature and two distinct maternal and fetal cell layers (Bridger, Menge, et al., 2007). It is in these sites that *S*. Dublin is thought to cross this placental barrier and cause abortion (Anderson, 2007). The concurrent production of angiogenic factors ensures the vascularisation of these tissues independently, and the formation of

the intertwining network of maternal and fetal blood vessels (Wooding and Burton, 2008). The placentomes are distinguishable and functional structures from around day 70 of gestation and continue to develop in complexity and number (Schlafer, Fisher, and Davies, 2000).

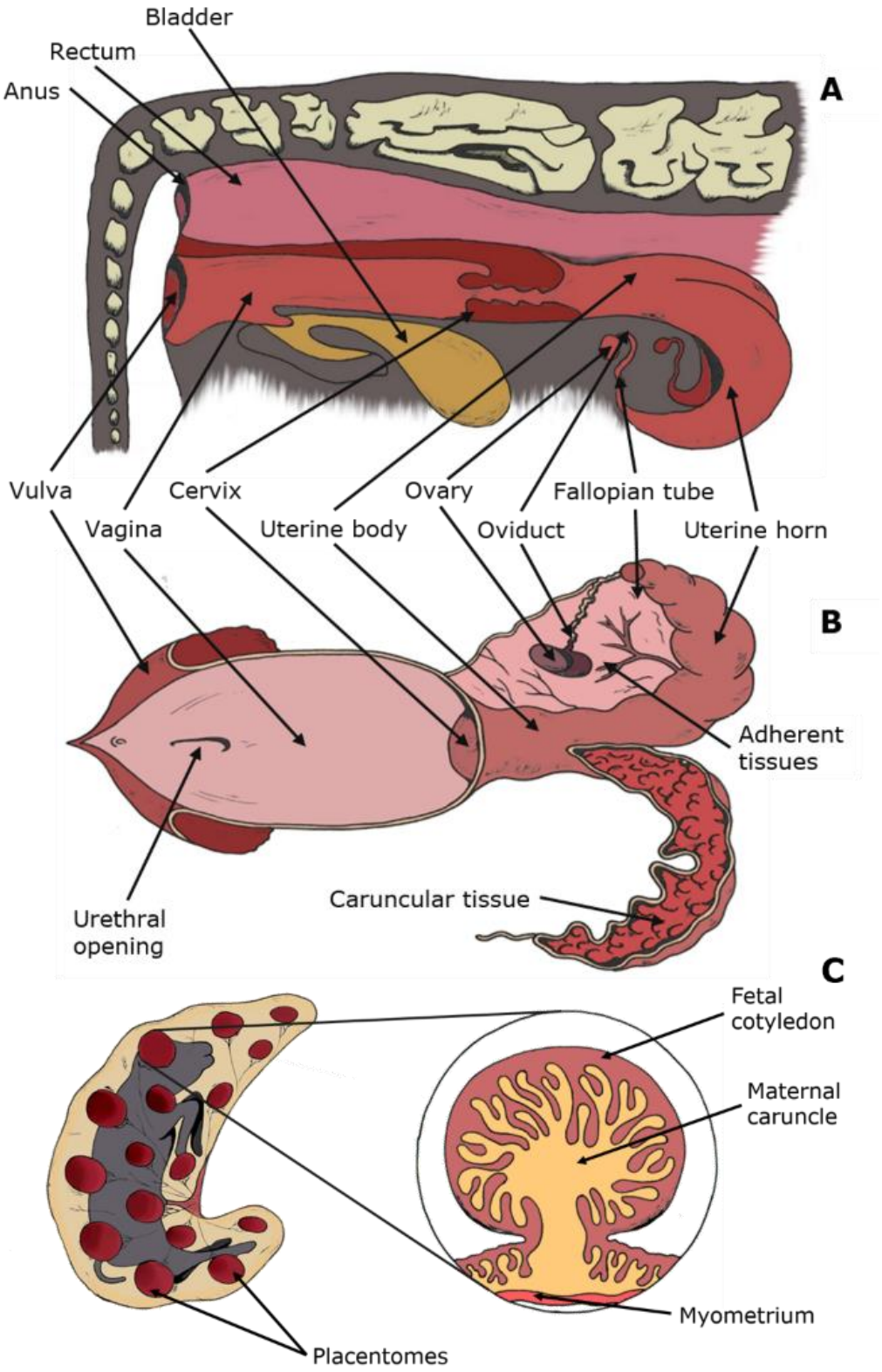


Figure 1.6 – Schematics of the bovine reproductive tract. A details the reproductive tract *in situ*, B shows the reproductive tract detailing the uterine horns and caruncular tissues, C details the situation of the placentomes around the fetus and the structure of the placentomes including the fetal cotyledonary and maternal caruncular tissues.

1.3.3 - Immunity during pregnancy

During pregnancy, the fetal allograft must be tolerated by the maternal immune system for the pregnancy to be successful. The concept of immune tolerance during pregnancy arose from the observation that women with cell mediated autoimmune disorders enter remission during pregnancy, whilst women suffering from lupus erythematosus experience more severe symptoms during pregnancy (Wegmann et al., 1993). Immune tolerance includes polarisation of the maternal immune responses towards an immunoregulatory Th2 response and away from a proinflammatory Th1 response which could be detrimental for the fetoplacental unit (Krishnan et al., 1996). Whilst the Th1/Th2 dichotomy emphasises the impact of T helper cells, the cytokines produced by these cells and associated with immunoregulatory versus proinflammatory responses are related. Th1 type cytokines include TNFα, CXCL8, IFNγ and IL-2, whilst Th2 type cytokines include IL-10, PGE_2 and TGFβ (Krishnan et al., 1996). Early studies investigating this Th1/Th2 dichotomy largely used resorption prone and resorption resistant mouse models. Mice prone to fetal resorption demonstrate that a strong Th1 bias in placental tissues is associated with pregnancy failure without any other stimulation (Tangri and Raghupathy, 1993). When infected with *Leishmania major* and *Toxoplasma gondii*, resorption-prone mice express TNF as part of this strong Th1 response which correlates to increased fetal resorptions compared to controls (Coutinho et al., 2012; Krishnan et al., 1996; Sousa et al., 2021). Administration of exogenous TNFα in both resorption prone and resorption resistant mouse strains increase the rate of fetal resorption, further demonstrating the deleterious impact of Th1 type cytokine expression on pregnancy in the mouse (Chaouat et al., 1990). In cattle, the presence of a Th2 type immune bias during pregnancy, characterised by elevated IL-10, has been identified but not fully characterised (Oliveira et al., 2013).

1.3.4 - Abortion

Abortions in cattle are typically described as the expulsion of a non-viable foetus before the 260th day of gestation, and after the 42nd day of gestation, or more than 24 hours before the expected date of birth (Hovingh, 2009). This differs from a "reproductive failure" or "stillbirth", both of which are categorised depending on the gestational age of the foetus. Reproductive failures occur before day 42 of gestation, whilst stillbirths are the expulsion of a foetus capable of independent life, but born dead (Carpenter et al., 2006; "Committee on Reproductive Nomenclature Recommendations for Standardising Bovine Reproductive Terms," 1972). Abortions can occur due to a variety of factors, including nutritional deficiencies, infectious diseases, environmental conditions and inappropriate animal management strategies (Barkallah et al., 2014). They can be sporadic, isolated incidences, or become "abortion storms" where a number of animals abort in a short period of time (Hinton, 1977). An abortion in a dairy herd in the UK has been estimated to cost around £630 (Cabell, 2007). These costs depend on the cost of replacement stock, milk prices, the gestational age of the foetus, and a number of other factors (Hovingh, 2009).

Of the different causes of abortion, the most commonly diagnosed are those pertaining to infectious diseases. The UK has historically suffered from Brucellosis, an infection caused by the bacterium *Brucella abortus* (Cutler, Whatmore, and Commander, 2004). *Brucella* usually causes abortion in the third trimester of pregnancy, and as such, causes serious losses on farm, as the cow cannot be brought back into oestrus fast enough to calf again that year (Silva et al., 2012). Due to surveillance strategies enforced by UK government, *Brucella* was eradicated in the UK in 1979 (DEFRA, 2004; Cutler, Whatmore, and Commander, 2004; DEFRA, 2004). This has highlighted the importance of other diseases, the most common being *Neospora caninum*, *Bacillus licheniformis, S.* Dublin, *Trueperella pyogenes* and BVD.

Neospora caninum is a protozoal parasite and causes sporadic abortions in cattle, usually in the second trimester (Anderson, 2007). *Neospora* has been known to cause abortion storms which can effect up to 60% of a herd (Haddad, Dohoo, and VanLeewen, 2005). Infiltration of large numbers of neutrophils are observed during infection with *Neospora caninum*, along with increases in proinflammatory mediators like CXCL8 and TNFα (Cantón et al., 2014b, 2014a; Jiménez-Pelayo et al., 2019b; Rosbottom et al., 2008). Infiltration of neutrophils is also observed in in abortions due to *Bacillus licheniformis,* a spore-forming bacterium thought to target the bovine placenta, resulting in abortion (Agerholm et al., 1999). *Trueperella pyogenes* is a bacterium which has been known to cause abortion in cattle in the UK but is most often associated with postpartum endometritis and reproductive losses related to this (Ponnusamy et al., 2017); (Bicalho et al., 2016). Bovine Viral Diarrhoea infections result in abortion in the first trimester which often goes undetected and is instead attributed to failure of conception or sub-optimal fertility (Kendrick, 1976). Necrotising placentitis and thrombosis is observed in abortion due to *B. licheniformis*, as well as BVD, *Brucella abortus* and *Chlamydia abortus* (Agerholm et al., 1999; Carvalho Neta et al., 2008; Kendrick, 1976; Sammin et al., 2006). It is unclear as to whether necrosis occurs because of the host response including infiltrating immune cells, or if this is due to the bacteria killing the cells. Necrosis in the placentome likely impacts the functionality of the placentome and therefore feto-maternal nutrient exchange and resulting in abortion (Entrican, 2002). However, the inflammatory response may also be essential to pathogen clearance, as is the case in *Listeria* and *Chlamydia* infection, where absence of inflammation can allow for the infection of the fetus (Barber, Fazzari, and Pollard, 2005; Entrican, Buxton, and Longbottom, 2001).

1.4 - *S.* Dublin as a cause of abortion

Research into *S.* Dublin as a cause of abortion is very limited, with few studies using models of disease or whole animals to investigate

the mechanisms behind abortion due to *S.* Dublin. Histopathological description of *S.* Dublin-induced abortion is also very limited.

Experimental infection with *S.* Dublin does not always induce an abortion, as was observed in pregnant cattle inoculated orally (Hall and Jones, 1979). In experimental infections with *S.* Dublin where animals were inoculated intravenously to deliberately induce an abortion, several animals did not abort and the researchers stated that, based on the histopathology, the animals were unlikely to have aborted (Hall and Jones, 1977). Some of this variation in clinical outcome is due to differences in inoculum, but variation between animals infected with the same bacterial dose (Hall and Jones, 1979). As all of the dams were inoculated with the same *S.* Dublin isolate, this suggests that the outcome of infection is highly dependent on the dam. However, other *S.* Dublin isolates with different virulence characteristics may have caused more severe disease and resulted in a higher proportion of animals aborting. Cattle in experimental studies with *S.* Dublin have also been reported to scour, a clinical sign not often associated with pathogenesis preceding abortion otherwise (Hall and Jones, 1979). This may have occurred due to the high doses used in the experiment.

Where clinical signs of disease and histopathology were observed, lesions were identified in the reproductive tissues including placentomes, ovaries and uterine wall, as well as the lung and liver (Hall and Jones, 1977). The development of lesions was apparent 2 days after inoculation, although this was the earliest time point in these experiments where lesions were identified (Hall and Jones, 1977). Neutrophilic placentitis is observed in *S.* Dublin abortions, similar to those of *Neospora caninum, Chlamydia abortus,* BVD and *Bacillus licheniformis* (Anderson, 2007; Hall and Jones, 1977). Additionally, degeneration of luteal cells was observed in aborting animals, but it is not clear as to whether this caused an abortion or was as a result of the abortion event (Hall and Jones, 1977). Foci of necrosis was frequently observed in the liver of animals which

aborted, including infiltration by neutrophils, macrophages and lymphocytes (Hall and Jones, 1977). Mild pathogenesis was observed in the lungs with small areas of neutrophilic congestion observed in the alveolar septae early during the onset of infection (Hall and Jones, 1977).

Altogether, it appears as though initial infection occurs and is systemically translocated through lymph nodes, the liver and lungs, before significant disease is observed in the placentomes preceding abortion. However, these studies are the only available whole animal studies which use pregnant cattle to observe the impact of infection on the outcome of pregnancy. The only other study investigating the impact of *S.* Dublin on the reproductive tract demonstrated the production of neutrophil chemoattractant CXCL8 in endometrial and intercotyledonary tissues, however this was in response to heat-killed *S.* Dublin isolates (Silva et al., 2012). The host response to the live bacterium, how this might impact the progression of abortion and whether the bacteria causes cell death in the placenta or whether an inappropriate response by the host causes placental necrosis is unclear.

1.5 - Summary

S. Dublin is swiftly becoming one of the leading causes of bovine abortion in the UK, presenting a threat to both animal and human health and welfare, and an economic burden to beef and dairy farmers.

The prevalence and importance of bovine abortions due to *S.* Dublin is likely highly underestimated, and as a result, this disease has become endemic on farms across the UK. With the dairy and beef industries seeing sharp declines in fertility, it is more important than ever to maintain the reproductive health of the livestock for which we are responsible. Concerns of *S*. Dublins zoonotic potential must be considered, particularly because of the severity of the disease it causes in humans. It is highly likely that abortions occur due to the presence of *S*. Dublin in the placentomes, often as a result of the

systemic infection of the dam. The use of a cell line derived from the placentomes will give a greater understanding of the interactions between the host and the bacterium, as well as determining the method of bacterial colonisation.

Investigation of *Salmonella enterica* serovar Dublin in the context of its abortogenic nature will aid in the identification of management strategies or interventions to prevent pregnancy loss in cattle due to this pathogen.

1.5.1 - Aims

The overarching aim of this thesis is to understand *S.* Dublin as a causative agent of abortion in cattle. To do this, the following areas will be addressed:

1. Characterise the causative bacteria and identify any potentially host-specific virulence factors associated with livestock infections of *Salmonellae.*
2. Determine bacterial virulence and survival in a host and tissue-specific model of the bovine reproductive tract and ascertain *S.* Dublin survival in whole blood to expand knowledge on methods of dissemination.
3. Investigate elements of the host response to *S.* Dublin in a model of the bovine reproductive tract and compare this to other, better understood abortifacient infections.
4. Identify antimicrobial resistance in *S.* Dublin with particular focus on antimicrobials used in livestock and compare this with antimicrobial resistance genes present in both *S.* Dublin and *S.* Typhimurium isolates.

Chapter 2 - Characterisation of clinical *Salmonella* Dublin isolates implicated in cases of bovine abortion in the UK in 2017

2.1 - Introduction

S. Dublin infections can be categorised into invasive (those causing systemic disease) and non-invasive (those limited to the gastrointestinal tract). Interestingly, in humans, different *S.* Dublin isolates from different countries appear to have different invasive potentials (Jones et al., 2008; Langridge, Nair, and Wain, 2009). The outcome of an infection is influenced by a variety of factors from both the host. In *S.* Dublin infection in cattle, host factors can include previous exposure and therefore immunity to the invading pathogen, current health and nutritional status and a variety of animal management factors like access to and quality of feed and environmental conditions (Anderson et al., 2001). However, it is likely that the differences between the invasiveness of *S.* Dublin isolates globally arise from both host factors and genomic factors impacting the virulence of the pathogen (Langridge, Nair, and Wain, 2009). The virulence of a pathogen can be described as the ability of the bacteria to invade host cells, survive and replicate in different host environments including acquiring different nutrients and bacterial motility (Holschbach and Peek, 2018).

The intracellular burden of *Salmonellae* has been linked to the intrinsic growth rates of *Salmonellae* which can in turn influence virulence and the outcome of infection (Grant et al., 2009). Bacteria with an intrinsically faster growth rate therefore could replicate and disseminate faster than those with intrinsically slower growth rates. Virulence genes *spvRABCD* located on a *Salmonella* virulence plasmid

were identified by researchers looking specifically at the impact of increased growth rates as a marker of virulence in *S.* Typhimurium isolates in mice (Gulig and Doyle, 1993). Conversely, a faster rate of replication may also elicit an immune response which clears the pathogen more rapidly, as is observed in cytosolic hyper-replication in *S.* Typhimurium (Knodler et al., 2010). Differences in growth rates could also impact the perceived invasiveness of an isolate when used in cell studies. For example, if one isolate is phenotypically less invasive compared to other isolates, the numbers of recovered bacteria after time in an infection model would be lower. However, if the same isolate had a faster growth rate, the number of intracellular bacteria would increase and potentially mask the impact of the reduced invasiveness. Therefore, determining the growth rates of isolates would improve the robustness of future experiments as well as identifying any isolates of interest.

As well as different host factors impacting the pathogenic outcome of an infection, some of the variation in clinical outcome (invasive versus non-invasive) will also be due to genetic variation among the population of *S.* Dublin isolates. This variation will be observed in the accessory genetic elements, as by definition these can differ between strains and are not necessarily essential for survival (Jacobsen et al., 2011; Moreno Switt et al., 2012). The *Salmonella* genomes can be described as being plastic, in that acquisition and loss of mobile genetic elements like plasmids leads to regular genomic change and adaptation (Aljahdali et al., 2020; Han et al., 2012). This plasticity is thought to be vital in the evolution and emergence of food-borne pathogens like *Salmonella* and *E. coli* the process of acquiring genetic material can lead to changes associated with virulence and fitness (Doublet et al., 2005). Furthermore, it is likely that the acquisition of different mobile genetic elements along with pseudogenisation of genes no longer required for pathogenicity is part of the divergence of different serovars into host specificities (Langridge et al., 2015; Wheeler, Gardner, and Barquist, 2018). The gastrointestinal microbiomes of mammals are known to be sources of mobile genetic

elements because they are already pre-colonised by commensal microorganisms. In the bovine rumen, horizontal gene transfer has been described in *Salmonallae* specifically, as well as in a host of other species (McCuddin et al., 2006; Smith, 1977). *Salmonellae* often cause inflammation in the gut which has also been proposed as a potential trigger for horizontal gene transfer (Stecher et al., 2012). Bacteria therefore may be more likely to acquire virulence genes associated with particular hosts simply because those genes are required to colonise that host and therefore more bacteria in the host niche are more likely to have these virulence genes. Virulence genes can also be acquired on plasmids which are selectively acquired because they convey resistance to particular antibiotics (Mangat et al., 2017). Antibiotics like tetracyclines and aminoglycosides are routinely purchased in the UK for use in livestock, so any virulence factors harboured on plasmids carrying resistance to tetracyclines or aminoglycosides could be co-selected for in livestock (Veterinary Medicines Directorate, 2020). Therefore, whilst many of the studies into *S.* Dublin specifically use isolates 2229 or 3246, the rate of genetic change and potential for the acquisition of different virulence-impacting genes highly favours the use of currently circulating, clinically relevant isolates (Betancor et al., 2012; Bolton et al., 1999; El Sayed et al., 2018; Hall and Jones, 1976, 1977, 1979; Hall et al., 1980; Heithoff et al., 2008; Olsen et al., 2013; Pullinger et al., 2008; Ung et al., 2019; Vohra et al., 2019).

Very few studies describe the growth and virulence of *S.* Dublin isolates, specifically from bovine sources. Those that do tend to focus on specific pathogenicity islands or mobile genetic elements, rather than looking more generally at virulence. As a result, there is a gap in the knowledge about *S.* Dublin virulence in UK isolates which could aid in our understanding of the molecular mechanisms underpinning *S.* Dublin infection and subsequent abortions. Additionally, describing the virulence factors present in isolates associated with bovine abortion could provide insights into potential vaccine targets or management strategies.

2.1.1 - Aims and hypotheses

This chapter aims to characterise the phenotypic growth of clinically relevant abortion-related isolates from the UK in 2017 and reference strain for use in future experiments. Comparing the growth patterns of these isolates will ensure that any differences can be accounted for in later experiments where growth could impact the outcome. Elucidating the MLST type of the isolates will demonstrate how representative they are of the UK population, currently dominated by sequence type 10. It will also be possible to distinguish any clonal isolates in this set, where their removal will reduce the amount of consumables and time required in future experiments. Finally, variation in virulence can be predicted by identifying the virulence factors present and this can also be accounted for in future experiments.

We hypothesise that there will be little to no difference in growth between the 15 clinical isolates from bovine abortions. However, there may be differences in the presence and absence of non-essential virulence factors between the 15 abortion isolates and reference isolate 2229 due to its long laboratory history.

2.2 - Materials and Methods

S. Dublin isolates from 15 cases of bovine abortion in the UK in 2017 were provided by the Animal and Plant Health Agency (APHA) and characterised alongside reference strain 2229, isolated from a case of salmonellosis in a calf in the UK (Baird, Manning, and Jones, 1985) (Table 2.1). These isolates were selected by the APHA and no meta-data were available, other than the year of isolation and that they were isolated from cases of bovine abortion.

Table 2.1 – 16 *S.* Dublin isolates used in this study. 15 were provided by the APHA and were isolated from cases of bovine abortion in 2017 in the UK. Isolate 2229 was provided by Paul Barrow at the University of Nottingham and was first isolated from a case of calf salmonellosis in 1985.

Isolate name	Provided by	Source
2229	Paul Barrow (University of Nottingham)	Baird, Manning, and Jones, 1985
L 1938/17	APHA – fetal stomach contents	Isolated from cases of bovine abortion in 2017
L 1941/17		
L 2100/17	APHA – bacterial, viral, fungal culture	
L 2104/17		
L 2135/17		
L 2160/17		
L 2162/17		
L 2185/17		
L 2284/17		
L 2294/17		
L 2348/17		
L 2424/17		
L 2469/17		
L 2517/17		
L 2591/17		

APHA – Animal and Plant Health Agency

2.2.1 - Isolate revival

Isolate 2229 was provided frozen in Microbank beads which were kept on ice during use and stored at -80°C (Pro-Lab Diagnostics). A single bead was removed from the Microbank, placed onto nutrient agar (NA) (Sigma Aldrich) and spread around on the agar plate using a sterile 10µl inoculation loop (Sigma Aldrich). All other isolates were

provided pre-cultured by the Animal and Plant Health Agency. Samples of these cultures were taken and spread onto NA using a 10µl sterile inoculation loop. Agar plates were incubated overnight in an air incubator at 37°C until colonies could be identified.

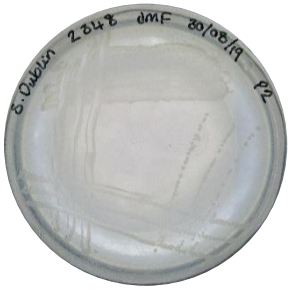
2.2.2 - Isolate storage

For the long-term storage of the isolates, three to four single colonies were picked from the plates described previously using a 10µl sterile inoculation loop and stirred into a Microbank vial (Pro-Lab Diagnostics). The Microbank vial was inverted three times and placed on ice before being moved to long-term storage at -80°C.

2.2.3 - *Salmonella* growth and selective media

All isolates were grown on Nutrient Agar (NA) (Sigma Aldrich, United Kingdom), MacConkey (Sigma Aldrich, United Kingdom) and Xylose Lysine Deoxycholate (XLD) agar (Sigma Aldrich, United Kingdom) to confirm the *Salmonella* phenotypes (Table 2.2). Isolates were streaked onto each type of agar using a 10µl inoculation loop and incubated at 37°C overnight in an air incubator. The colony morphology of each isolate was also observed on NA, where the individual describing the colonies was unaware of the isolate identity to eliminate bias.

Table 2.2 – Agars used to observe colony morphology and phenotype of *S.* Dublin isolates, including selection or indicator where appropriate. Isolates were streaked onto each agar using a 5µl inoculating loop and incubated overnight at 37°C.

Agar	Selection/Indicator	Description	Image
Nutrient	None	Small, opaque colonies	
MacConkey	Lactose fermenting bacterium give rise to yellow rings around colonies. Non lactose fermenting bacterium do not change colour of agar.	Lactose-fermenting bacterium turns plate yellow. Colonies are pale.	
Xylose Lysine Deoxycholate (XLD)	Selects for *Salmonella* and *Shigella* spp which give rise to red colonies. Metabolism of hydrogen sulphide forms black colonies in *Salmonellae* to differentiate.	Black precipitating colonies specific for *Salmonella* phenotype.	

2.2.4 - Determination of *S.* Dublin Exponential Growth Rates and Doubling Times

OD is proportional to the number of bacterial cells within log phase. The *S.* Dublin isolates were grown overnight to stationary phase in NB in an orbital shaking incubator (Thermofisher) at 37°C and 150RPM. 100µl of these cultures were "washed" by centrifuging at 17000*g* for 10 minutes to form a pellet. The supernatant was removed, and the pellet was resuspended in 100µl sterile phosphate

buffered saline (PBS). The samples were washed again and 1ml of either PBS, NB or Dulbecco's Modified Eagle's Medium/Ham's F-12 (Corning Media Tech, UK), supplemented with 10% fetal calf serum (FCS) and 10mg/ml L-glutamine (DMEM). This DMEM was used as an antibiotic-free cell culture medium, modelling conditions in future experiments. 200µl was taken from each of the cultures and added to wells of a 96-well plate. Growth was measured by absorbance (au) at 612nm over ten hours in a TECAN Genios Pro 96/384 Multifunction Microplate Reader, incubated at 37°C.

Logistic growth curves were fitted to the data in GraphPad Prism (V 9.5.1) which identifies the maximal growth rate as k (Equation 2.1) (Hotson and Schneider, 2015). The doubling time was calculated for each isolate (Equation 2.2, from Norris et al., 2020). Growth experiments were performed four times and the growth rates and doubling times were calculated for each of the four experimental repeats in NB and DMEM.

$$Y = \frac{YM * Y0}{((YM - Y0) * \exp(-k * x) + Y0)}$$

Equation 2.1 – Model for fitting logistic growth curves to *S.* Dublin isolates used by GraphPad Prism. Where: Y0 = starting population; YM = maximum population; k is the rate constant; 1/k = the x coordinate of the first inflection point.

$$Dt = \ln(2)/r$$

Equation 2.2 – Calculation to find the doubling time of *S.* Dublin isolates. Where: Dt = doubling time; r = rate (calculated as the slope value of the exponential section of growth curves under linear regression analysis).

2.2.5 - DNA Extraction

The isolates were grown overnight in NB in an orbital shaking Incubator (Thermofisher) at 37°C, ensuring cultures were in stationary phase after exhausting nutrients supplied. DNA of 1ml of culture was extracted using the QiAmp DNA Mini Kit (QUIAGEN), as per the manufacturer's instructions. Briefly, 1ml of the bacterial culture was centrifuged at 5400*g* for 5 minutes and suspended in 180µl of buffer ATL. 20µl of mAU/ml Proteinase K was added to each

sample and incubated at 56°C for 30 minutes to lyse the bacteria. 200µl Buffer AL was added, and the samples were pulse vortexed for 15 seconds before being incubated at 70°C for 10 minutes. 200µl of 100% ethanol was added to adjust the DNA binding, and the samples were added to QIAGEN Spin Columns to be centrifuged at 6200*g* for 1 minute. The filtrate was discarded, and the Spin Column was placed into a new Collection Tube. 500µl Buffer AW1 was added and centrifuged at 6200*g* for 1 minute. The Spin Column was placed into a new Collection Tube, and the filtrate discarded. 500µl Buffer AW2 was added and centrifuged at 16200*g* for 3 minutes. The Spin Column was placed into a sterile 1.5ml centrifuge tube, and filtrate was discarded. The Spin Column and 1.5ml centrifuge tube were then centrifuged at 17000*g* to dry the membrane. 100µl sterile molecular-grade water was added to the column and centrifuged at 17000*g* for 1 minute. A further 100µl of sterile molecular-grade water was added to the column and centrifuged at 17000*g* for 1 minute to give a final volume of 200µl.

2.2.6 - DNA Quantification

DNA was quantified using the Invitrogen Qubit dsDNA High Sensitivity Assay Kit according to the manufacturer's instructions. 10µl of Qubit Standard 1 and 2 was added to 190µl of the Qubit Working Solution in Qubit Assay Tubes and vortexed for 3 seconds. The standards were incubated at room temperature for 2 minutes. Standard 1 and 2 were read in the Qubit Fluorometer to calibrate the system. 2µl of each DNA sample was added to 198µl Qubit Working Solution in Qubit Assay Tubes and vortexed for 3 seconds. The samples were incubated at room temperature for 2 minutes. Each sample was read in the Qubit Fluoremeter to find the concentration of genomic DNA in each sample (Appendix Table 9.2). Extracted DNA was frozen at -20°C, before being moved to -80°C for longer-term storage.

2.2.7 - Whole-Genome Sequencing and Alignment

Illumina Short Read sequencing was performed by MicrobesNG. Multilocous Sequence Typing (MLST) analysis was performed by Dr

Adam Blanchard (University of Nottingham) using ChewBBACA based on a 95% loci presence (Silva et al., 2018). Genome alignment was completed using Roary based on core and accessory genes by Dr Adam Blanchard, and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015).

2.2.8 - Identification of Virulence Factors

Virulence factors were identified by comparing the whole genome sequences of the isolates to the sequences of known virulence factors of pathogenic bacteria within the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). The algorithm used by the VFDB to assess gene presence is not well described but has been used previously in similar studies (dos Santos et al., 2021; Yan et al., 2022). The identification of virulence determinants was performed by Dr Adam Blanchard (University of Nottingham). As part of the data cleansing process, genes with <90% identity were considered "absent" and were excluded from the analysis. Genes with ≥90% identity were described as "present" similar to other studies in *Salmonella* (González-Torres et al., 2023; Mu et al., 2022). Isolates were categorised into different virulence profiles based on the presence and absence of the virulence genes. To discern the functions of each virulence gene, the gene name was queried in VFDB, NCBI Gene (National Library of Medicine and National Center for Biotechnology Information, 2004) and NCBI Protein (National Library of Medicine and National Center for Biotechnology Information, 1988). Where records were "discontinued" in NCBI Gene, the "CoDing Sequence" (CDS) tag was identified and a search was performed in NCBI Protein to find current information for the gene of interest. If required, gene names were updated to reflect recent advancements and include the most up-to-date information. Genes were grouped based on their functionality and variation within these groups between virulence profiles was identified. If variation was observed in any genes within a group, the group was deemed "variable".

2.2.9 - Statistical analysis

Kruskal-Wallis and Dunn's multiple comparisons tests were used to test for differences between values within the exponential growth rate and doubling time data. One Sample Wilcoxon tests were performed to discern any differences between time points in these data sets. Statistical tests were performed using GraphPad Prism 8.1.

2.3 - Results

Bacterial virulence can be impacted by a range of different parameters which in turn can influence the outcome of infection. Prior to the use of the 16 isolates described in further studies, it was important to characterise these bacteria first. The growth of 15 abortion-associated isolates and a reference strain of *S.* Dublin was investigated and genotypic analysis was performed to identify virulence factors.

2.3.1 - Phenotype of *S.* Dublin Isolates

All isolates exhibited the expected *Salmonella* spp. phenotype on MacConkey (pale colonies and fermentation causing agar to yellow) and XLD (pale colonies with black precipitate formation) agars. Five isolates (2229, L 2185/17, L 2294/17, L 2424/17 and L 2591/17) displayed a mucoid colony growth and isolate L 2185/17 grew with a "rough" colony morphology without a clear circular edge on NA (Figure 2.1).

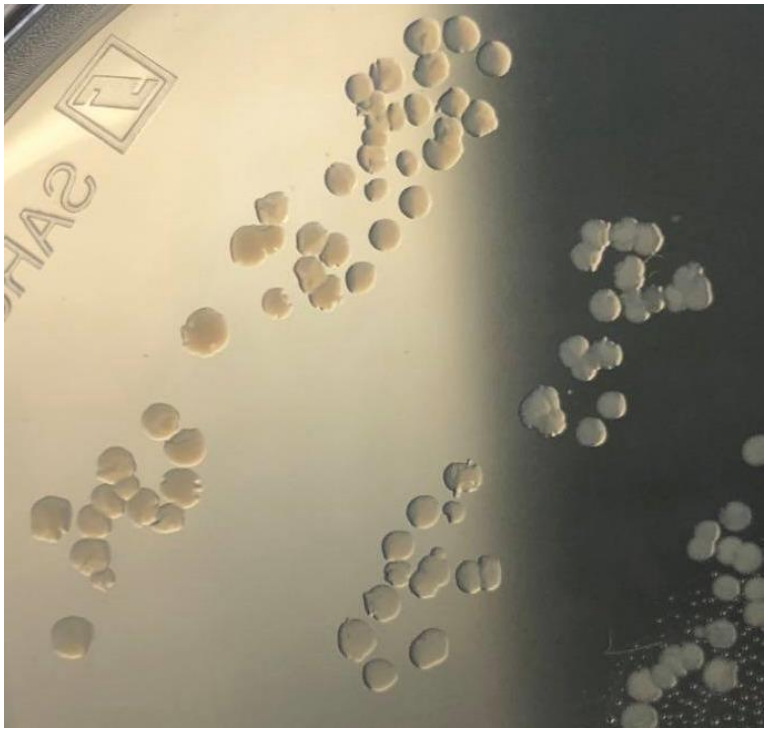


Figure 2.1 - "Rough" colony morphology observed in *S.* Dublin isolate L 2185/17 on nutrient agar. Isolates were streaked onto NA and incubated overnight in an air incubator at 37°C.

2.3.2 - Exponential Growth Rate and Doubling Time

It was important to identify exponential growth rates and doubling times of the isolates in NB and DMEM in order to use these isolates in future experiments. PBS was used as an osmotically balanced control media in which bacteria can survive but do not replicate due to a lack of available nutrients.

Growth was measured in absorbance (au) at 612nm over the course of 10 hours. As expected, isolates did not grow in PBS. All isolates other than L 2185/17 showed a characteristic pattern in their growth in NB and DMEM, which was reproducible across the four replicate experiments (Figure 2.2 and Appendix Figure 9.2, Figure 9.3, Figure 9.4, Figure 9.5, Figure 9.6, Figure 9.7 and Figure 9.8).

Similar growth patterns were observed in NB and DMEM across all of the isolates and there were no significant differences between the growth rates of any of the isolates in either DMEM or PBS (Table 2.3 and Table 2.4). Growth rates in DMEM ranged from 0.004557 min^{-1}

to 0.008277 min^{-1}, whilst in NB these ranged from 0.0028143 min^{-1} to 0.014755 min^{-1} (Table 2.3 and Table 2.4). Doubling times overall ranged from 90.18 min to 166.25 min in DMEM and 71.84 min to 311.21 min in NB (Table 2.3 and Table 2.4).

Isolate L 2185/17 consistently displayed biphasic growth patterns across the four replicate experiments despite its growth varying considerably (Figure 2.2 E-H). This biphasic pattern was characterised by an initial period of growth followed by either a period of consistent absorbance or decrease and final phase of growth. The growth rates of each of the phases did not differ significantly from those of any other isolate in either media. The doubling times observed in phase 1 of isolate L 2185/17 in NB differed significantly to the doubling times of isolates L 2348/17 ($p = 0.0407$), L 2424/17 ($p = 0.0455$) and L 2469/17 ($p = 0.0363$) in DMEM (Table 2.3 and Table 2.4).

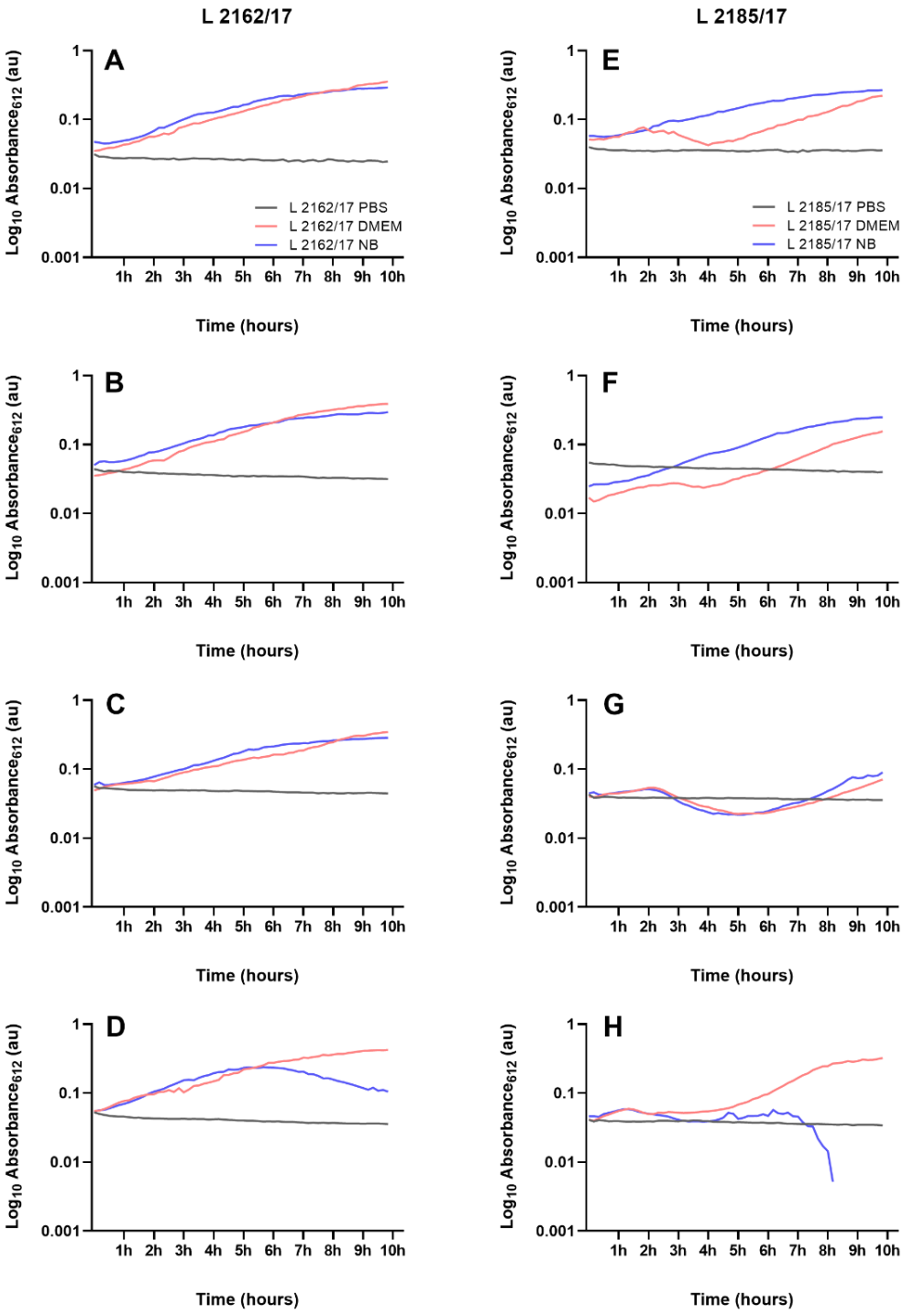


Figure 2.2 - Growth of *S.* Dublin isolates L 2162/17 and L 2185/17 in PBS, BCECM and NB. Isolates were grown in phosphate buffered saline (PBS), Bovine Caruncular Epithelial Cell culture Medium (BCECM) without antibiotics and nutrient broth (NB) for ten hours at 37°C, represented by grey, red and blue lines respectively. Absorbance at wavelength 612nm was measured every ten minutes. Each isolate was analysed four times. Isolate L 2162-17 (A-D) represents the general growth pattern shown by all isolates, whilst L 2185-17 (E-H) appears to exhibit a biphasic growth pattern.

Table 2.3 - Growth rates and doubling times of S. Dublin isolates grown in DMEM. Isolates were grown in Bovine Caruncular Epithelial Cell (BCECs) culture medium without antibiotics (DMEM) over the course of ten hours at 37°C. Absorbance readings at 612nm were taken every ten minutes. Exponential growth rates were determined by fitting logistic growth curves to absorbance readings, and doubling time was calculated using Equation 2.2. Isolate L 2185/17 exhibited biphasic growth, so each phase was identified and analysed separately, highlighted as 1 and 2.

Isolate	Growth Rate ± Standard Deviation (min^{-1})	Doubling Time ± Standard Deviation (min)
L 1938/17	0.0064 ±0.0014	114.73 ±31.29
L 1941/17	0.0061 ±0.0015	120.61 ±32.33
L 2100/17	0.0051 ±0.0007	139.94 ±19.42
L 2104/17	0.0050 ±0.0016	155.29 ±50.41
L 2135/17	0.0063 ±0.0016	120.84 ±40.19
L 2160/17	0.0063 ±0.0021	126.36 ±50.32
L 2162/17	0.0057 ±0.0015	131.96 ±42.17
L 2185/17[1]	0.0058 ±0.0030	156.38 ±80.13
L 2185/17[2]	0.0065 ±0.0023	117.04 ±29.85
L 2284/17	0.0083 ±0.0072	137.98 ±65.34
L 2294/17	0.0072 ±0.0036	121.09 ±55.82
L 2348/17	0.0047 ±0.0011	158.89 ±43.95
L 2424/17	0.0047 ±0.0011	154.58 ±35.78
L 2469/17	0.0046 ±0.0014	166.25 ±48.03
L 2517/17	0.0049 ±0.0012	149.31 ±29.86
L 2591/17	0.0054 ±0.0018	144.66 ±47.63
2229	0.0081 ±0.0020	90.18 ±19.93

Table 2.4 - Growth rates and doubling times of S. Dublin isolates grown in NB. Isolates were grown in nutrient broth (NB) over the course of ten hours at 37°C. Absorbance readings at 612nm were taken every ten minutes. Exponential growth rates were determined by fitting logistic growth curves to absorbance readings, and doubling time was calculated using Equation 2.2. Isolate L 2185/17 exhibited biphasic growth, so each phase was identified and analysed separately, highlighted as 1 and 2.

Isolate	Growth rate ± Standard Deviation (min^{-1})	Doubling Time ± Standard Deviation (min)
L 1938/17	0.0106 ±0.0054	78.04 ±25.27
L 1941/17	0.0112 ±0.0051	71.84 ±21.78
L 2100/17	0.0105 ±0.0052	78.12 ±24.65
L 2104/17	0.0103 ±0.0049	79.65 ±26.99
L 2135/17	0.0110 ±0.0047	72.36 ±21.73
L 2160/17	0.0110 ±0.0053	77.18 ±30.72
L 2162/17	0.0102 ±0.0052	81.39 ±26.17
L 2185/17[1]	0.0028 ±0.0013	311.21 ±149.24
L 2185/17[2]	0.0069 ±0.0009	102.35 ±13.45
L 2284/17	0.0148 ±0.0127	75.77 ±34.02
L 2294/17	0.0098 ±0.0056	87.31 ±30.08
L 2348/17	0.0097 ±0.0057	89.79 ±32.36
L 2424/17	0.0103 ±0.0054	81.25 ±26.64
L 2469/17	0.0104 ±0.0058	82.60 ±28.34
L 2517/17	0.0104 ±0.0057	82.95 ±29.83
L 2591/17	0.0106 ±0.0057	79.34 ±26.64
2229	0.0107 ±0.0055	78.55 ±27.38

2.3.3 - Investigation of the similarity of *S.* Dublin isolates using MLST and phylogenetic alignment

Phylogenetic alignment of the 16 isolates allowed for the investigation of the isolates similarity, as well as identifying any clonal isolates.

All isolates were found to have MLST sequence type 10 and fall into three distinct phylogenetic clades (Figure 2.3). The three clades were defined as such because there were three branches emanating from the root of the tree (Appendix Figure 9.9). The smallest clade included one isolate (L 2591/17), followed by a clade of three isolates (L

2160/17, L 2424/17 and L 2469/17). The largest clade contained the remaining twelve isolates, including reference strain 2229. Branch lengths indicated that none of the isolates were clonal.

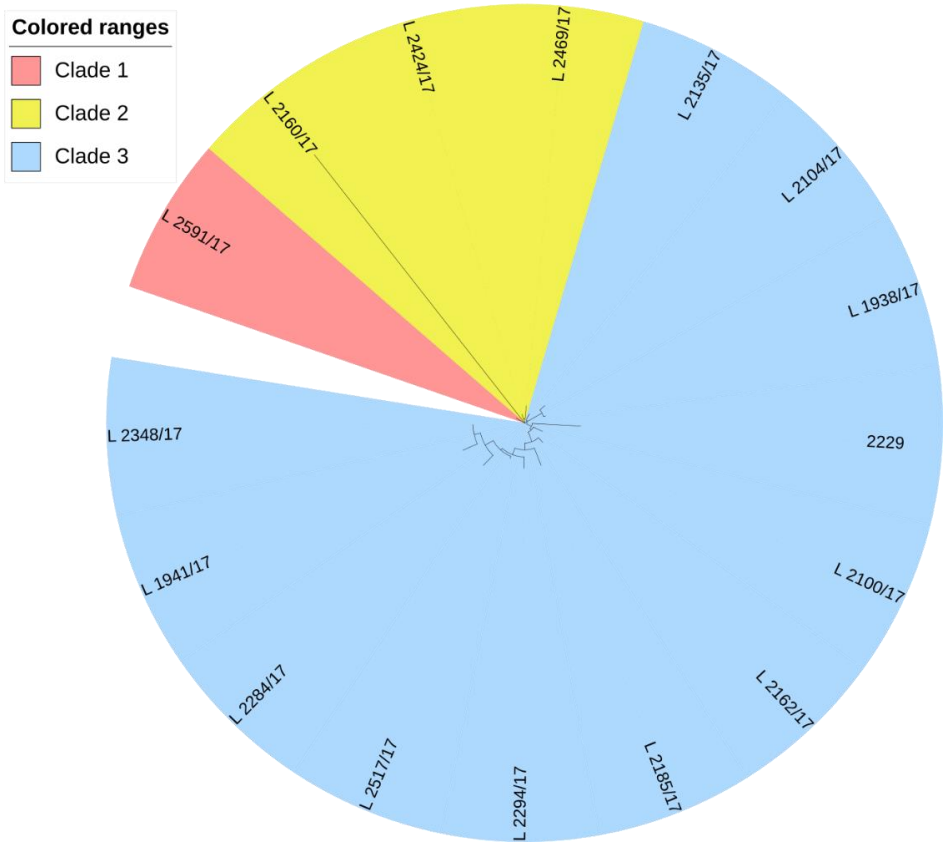


Figure 2.3 - Phylogenetic alignment of 16 *S.* Dublin isolates based on Whole Genome MLST. Whole genome Multi-Locus Sequence Type (MLST) was completed on a 95% loci presence, aligned using Roary by Dr Adam Blanchard, and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015). Clades were defined as such because three branches emanated from the root of the phylogenetic tree (Appendix Figure 9.9).

2.3.4 - *S.* Dublin virulence factors

Virulence factors are essential for bacterial pathogenesis and may differ between bacterial isolates in the same species. Therefore, it was important to investigate the presence and absence of virulence factors of the 16 *S.* Dublin isolates (Table 2.1) that would be used in future host infection work to determine if there were any differences between isolates which could impact those results.

A total of 238 different virulence genes were identified among the 16 *S.* Dublin isolates associated with virulence functions including adherence, motility, metabolism and secretory systems (Appendix Table 9.3). These virulence genes were categorised into 19 different functional groups (Figure 2.4). Genes associated with two different Secretion Systems were identified, including T6SS (n=26), and T3SS which was further classified into SPI1 (n=41), SPI2 (n=35) and both (Figure 2.4). Other groups identified were adherence (n=4), outer membrane proteins (n=2), chemotaxis (n=7), fimbriae (n=58), curlin (n=4), flagella (n=42), ferric uptake (n=6), magnesium transport (n=2), vi antigen (n=3), toxin (n=3) and antimicrobial resistance (n=1) (Figure 2.4). Two copies of SPI2 gene *sseJ*, T6SS genes *hcp* (also known as *tssD*)*, tssC*, *tssF*, *tssG, tssJ* and *tssK* and fimbrial genes *stiA* and *stfG* were present in all 16 isolates, totalling 244 genes in all but one isolate. 242 virulence genes in total were identified in isolate L 2160/17. From this, 3 VPs were identified – VP1 was shared by 14 isolates whilst VP2 (reference isolate 2229) and VP3 (isolate L 2160/17) represented one isolate each. The three VPs differed by the presence or absence of three virulence genes – *stfE, fimW,* and *ssaU*.

Of the SPI1 genes identified, eight were categorised as secreted effector proteins (*avrA, orgC, sipA, sopA, sopB, sopD, sopD2, sptP* and *spvB*), seven as regulatory factors (*hilA, hilC, hilD, invF, invJ, rpoS* and *spvR*), five as chaperones (*iacP, invB, invE, sicA* and *sicP*) and 19 as structural proteins involved in needle complex and pore formation (*invA, invG, invH, invI, orgA, orgB, prgH, prgI, prgJ, prgK, sipB, sipC, sipD, spaO, spaP, spaQ, spaR, spaS* and *spvA*). SPI2 genes were similarly categorised into twelve secreted effector proteins (*sifA, sifB, sopE, sseE, sseF, sseI, sseJ, sseK1 sseK2, sseL, sspH2,* and *steC*), two regulatory proteins (*ssrA* and *ssrB*), four chaperones (*ssaH, sscA, sscB* and *sseA*) and 17 structural proteins (*spiC, ssaD, ssaE, ssaI, ssaJ, ssaK, ssaL, ssaM, ssaN, ssaP, ssaQ, ssaR, ssaT, ssaU, ssaV, sseC,* and *sseD*). All SPI1 and SPI2 genes encoding effector proteins were present in all of the isolates. The sequence of structural gene *ssaU* was less than 90% similar in isolate L 2160/17 and this

gave rise to VP3. Twelve of the thirteen "core" genes were present among the 26 T6SS genes identified (*tssA, tssB, tssC, tssD* (also known as *hcp*), *tssE, tssF, tssG, tssH, tssJ, tssK, tssL* and *tssM*) whilst *tssI* (also known as *vgrG*) did not meet the identity threshold of >90% (identity = 77.95%) (Cianfanelli, Monlezun, and Coulthurst, 2016).

Among the 42 different flagellar genes identified, the major structural genes comprising the flagellar hook and basal body were all present (*flgB, flgC, flgE, flgF, flgG, flgH, flgI, flgK, flgL, flhA, flhB, fliE, fliF, fliG, fliH, fliI, fliJ, fliM, fliN, fliO, flip, fliQ, fliR, motA* and *motB*) along with genes associated with regulatory functions (*flgA, flgM, flhC, flhD, flhE, fliA, fliB, fliK, fliL, fliS, fliT, fliY* and *fliZ*) and genes associated with non-structural accessory proteins (*flgD, flgJ, flgN* and *flk*). Genes encoding the flagellar filament (*fliC* and *fliD*) were not identified in this study. Elements of 14 different fimbrial gene clusters were present amongst the 49 fimbriae genes, representing five different fimbrial chaperone-usher clades, γ-1 (*bcf, fim, lpf, sth* and *sti*), γ-3 (*saf* and *sef*), γ-4 (*peg, yeh,* and *stb*), π (*std, ste* and *stf*) and κ (*pef*). *yeh* is orthologous in *Escherichia coli* as *peg* in *Salmonella spp.* The only "intact" fimbrial gene clusters were *sti* and *lpf*, intact referring to clusters observed in other *Salmonella* serovars. Genes encoding adhesins were present in eight of the gene clusters (*bcfA, fimH, sthE, stiH, sefD, pegD, stbD* and *safD*) but absent in three gene clusters (*steG, stfH* and *pefD*). The adhesin of the *std* fimbrial cluster is currently not available for categorisation but was previously thought to be *stdD* which was identified in this study but has only "discontinued" records for categorisation. Variation in the presence or absence of fimbrial genes gave rise to two virulence profiles – VP1 has *fimW* whilst VP2 does not, and VP2 has *stfE* whilst VP1 does not.

Four members of the *csg* curli gene cluster, including *csgA, csgB, csgE* and *csgG* were present. *csgA* and *csgB* are usually clustered in *Salmonellae* in the *csgBAC* operon and *csgE* and *csgG* are usually clustered as *csgGEFD* in *Salmonellae* (Barnhart and Chapman, 2006; Römling et al., 1998). Similarly, genes encoding and controlling vi

antigen expression were present in these isolates but were incomplete operons (*tviD, tviE* biosynthetic genes from *tviABCDE* and *vexB* from antigen export operon *vexABCDE*) (Hashimoto et al., 1993). The iron uptake operon *iroBCDEN* was present in all of the isolates along with ferric uptake regulator *fur* (Troxell et al., 2011).

Two mobile genetic elements were identified, with *grvA* from *Salmonella* Phage 19 and *mig-5* associated with plasmid pSENV (Figure 2.4). A further 12 genes were identified as virulence factors but their records were either hypothetical or discontinued (n=7) or "domain of unknown function" (DUF) proteins (n=4) including a dcrB-like protein (Appendix Table 9.3). *stdD* was also identified but there were no records available on the databases used (Figure 2.4). 7 of the identified virulence factors were present in two copies (T6SS genes *tssC*, *tssF*, *tssG*, *tssJ*, *tssK* as well as fimbrial gene *stfG* and SPI-2 gene *ssaJ*) (Appendix Table 9.3).

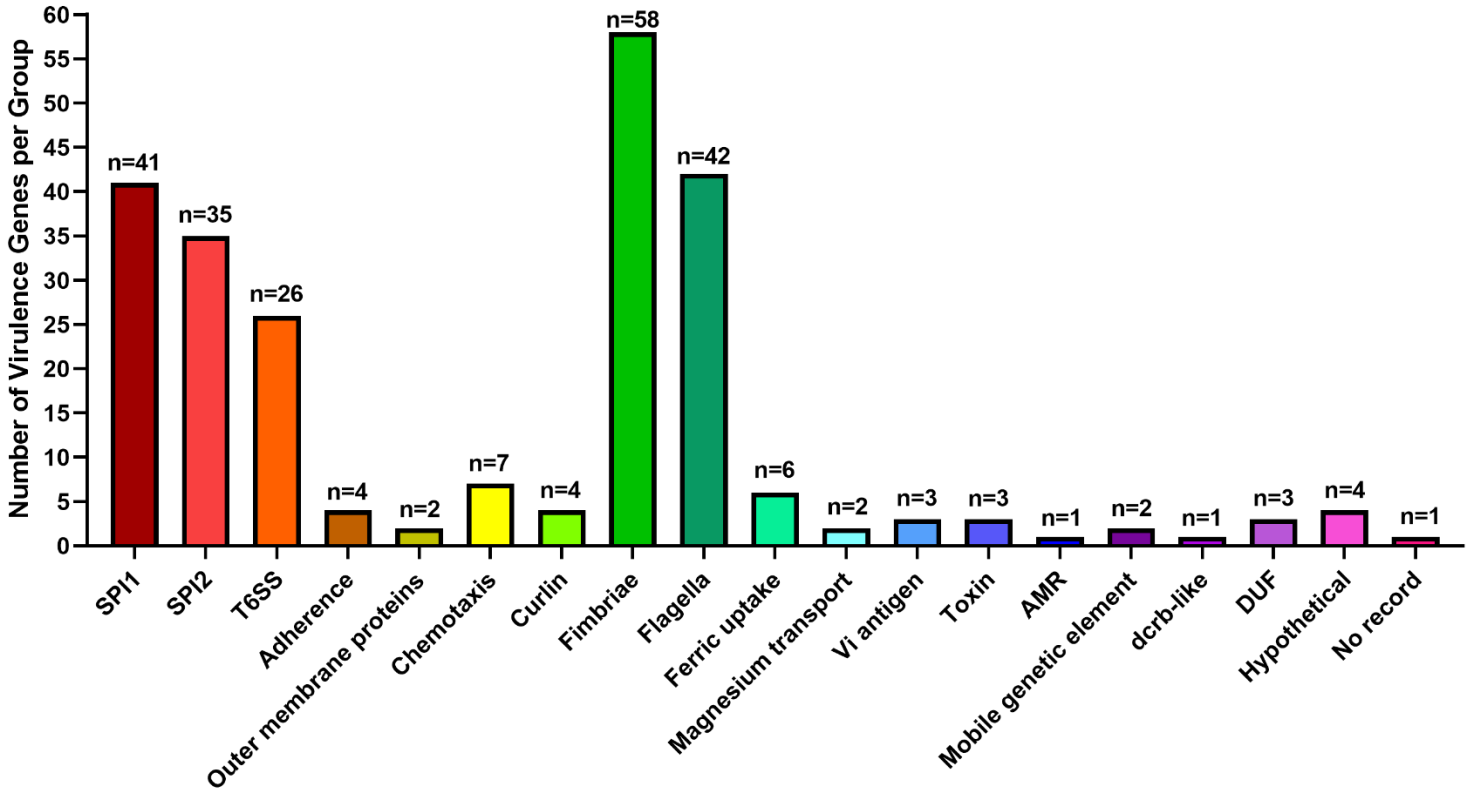


Figure 2.4 - Number of virulence factors within each of the 22 categories identified in 16 *S.* Dublin isolates. Virulence factors were identified by comparing the whole genome sequence of each isolate to a known reference in the Virulence Factor Database (Liu et al., 2019) by Dr Adam Blanchard (University of Nottingham). Genes below 90% identity were removed from the analysis. Investigation of the function of each virulence factor was completed using NCBI Gene and NCBI Protein and genes were grouped based on these functions. The number of genes in each functional group is indicated. Duplicates of the same gene are included in the total number of genes. Total number of virulence genes = 244. Total number of different virulence genes = 238.

2.4 - Discussion

Very little variation was observed between the 16 *S.* Dublin isolates in all phenotypic and genotypic analyses conducted. This was to be expected as all of the isolates were identified in clinical illness in cattle and therefore should share similar, if not the same capabilities in causing disease. This includes growth and virulence traits.

It was important to assess the isolates growth prior to their use in future studies, where differences could impact the outcome of an experiment. The isolates showed the expected phenotypes characteristic of *Salmonella* spp on MacConkey and XLD agar and confirmed that the cultures were not contaminated with any other bacteria. Whole genome sequencing also confirmed that these isolates were *S.* Dublin. Isolate L 2185/17 showed marked differences in its phenotype compared to all other isolates, including the reference isolate 2229. The phenotypic growth rates of all isolates except for L 2185/17 were highly reproducible across replicate experiments, and the experiments conducted allowed the identification of an appropriate time for incubation in which growth enters logarithmic phase for future work. The "rough" colony morphology observed in isolate L 2185/17 may also be indicative of this biphasic growth pattern as seen in other bacterial species (Neysens et al., 2003). Reductions in absorbance observed in isolate L 2185/17 as part of its biphasic growth pattern may be due to a proportion of the bacteria undergoing autolysis. This biphasic or diauxic growth may occur due to the microorganism undergoing a metabolic switch, using one nutrient source and then another if the first has been exhausted (Baker, Griffiths, and Nicklin, 2011). It is possible that having grown the bacteria overnight in NB, inoculating DMEM with the bacteria could cause a preferential switch in nutrient metabolism for a nutrient available in DMEM but not in NB. This specific isolate may be unable to make this switch as quickly and efficiently as other isolates. This metabolic switch would likely be mediated by genetic factors which could ultimately be investigated using whole genome sequencing and the identification of metabolic

genes that differ between genes in isolates that do not undergo this switch. For subsequent work with these isolates however, this information was not necessary. The significant difference observed between the doubling times of isolate L 2185/17 in growth phase 1 in DMEM and the doubling times of three other isolates in NB is not important for future work as all of the isolates would be grown in the same media at the same time.

The predominant *S.* Dublin MLST type in the UK and globally is ST10, but many other STs have been identified in *S.* Dublin, including ST73, 2037, 1552, 1487 and 1494 (Achtman et al., 2012; García-Soto et al., 2021; Lupolova et al., 2017; Mohammed et al., 2017; Manal, Mohammed and Thapa, 2020; Paudyal et al., 2019; Vilela et al., 2020; Zeinzinger et al., 2012). Therefore, it was to be expected that the isolates in this study were found to be ST10. Phylogenetic alignment of the isolates reveals their high degree of similarity, as has been reported previously, whilst indicating that none of the isolates are clonal (Langridge et al., 2015).

The high level of similarity was also observed in the analysis of the different virulence factors present in the 16 isolates. This too was to be anticipated as all the isolates in this study were collected from cases of bovine disease, so all isolates should have been capable of causing a clinical infection in cattle. All of the isolates should logically have the necessary virulence determinants required to infect a bovine host and would therefore be highly similar. This cannot be assumed however, as the isolation of the bacterium does not necessarily ensure that the pathogen is virulent and could be collected accidentally due to contamination or co-infection. Differences in virulence then may only be highlighted upon analysis of virulence factors or if isolates were used in infection models (Blanchard et al., 2020).

Homologous sequences for the SPI-2 virulence gene *ssaU* were not identified in isolate L 2160/17, denoted by the fact that there was no identity score available for this gene in this isolate. However, it is extremely unlikely that *ssaU* was absent in this isolate because it is a

key part of the SPI2 secretion system and essential for intracellular survival. SsaU is a structural component of the SPI-2 T3SS and, like many other components of T3SS, is highly conserved across different bacterial lineages (Deng et al., 2017; Dietsche et al., 2016). The deletion of *ssaU* in *S.* Typhimurium prevents the secretion of effector and translocon proteins essential for bacterial survival (Yu et al., 2018). In *S.* Typhimurium, mutations in the SPI-1 T3SS *spaS* homologue of *ssaU* impacted the secretion hierarchy and decreased secretion of effector proteins like SptP (Feria et al., 2015). Therefore, it does not make biological sense that an assumed virulent strain isolated from a case of bovine abortion would not have a functional SPI2 secretion system. This may demonstrate a limitation of the VFDB and suggests that there was no record of a heterologous yet functional *ssaU* gene similar to that present in the *S.* Dublin isolate L 2160/17 at the time of analysis. As *S.* Dublin is a highly under-researched serovar, many of the genes in the VFDB are those found in *S.* Typhimurium, which may not be comparable due to host and niche specificities of these different serovars.

Similarly, the absence of fimbrial gene *fimW* in isolate 2229 and its impact on pathogenicity cannot be ascertained because this isolate was still implicated in bovine salmonellosis. FimW is an autoregulator of the expression of type 1 fimbriae which aid in host-cell adhesion and is activated by FimY and FimZ (Saini, Pearl, and Rao, 2009). Studies in *S.* Typhimurium have demonstrated that mutations in *fimW* increase fimbrial expression and haemagglutination because of this elevated fimbrial expression (Tinker, Hancox, and Clegg, 2001). Whilst fimbrial expression is important for cellular adhesion and invasion, fimbriae are also known to activate host inflammatory responses which can lead to pathogen clearance (Kuzminska-Bajor, Grzymajlo, and Ugorski, 2015). Therefore, the virulence of isolate 2229 could be different to that of the other isolates in this study. Alternatively, isolate 2229 may have a heterologous sequence for *fimW* which was not present in the VFDB at the time of analysis. Fimbrial gene *stfE* was only identified in isolate 2229, although the

remainder of the *stfACDEFG* operon was not present in this isolate. Variation in this operon was to be expected as previous work with *S.* Typhimurium isolates deficient in different fimbrial operons showed that the *stf* operon is not essential for intestinal persistence in mice (Weening et al., 2005).

Few studies have been conducted into the virulence factors of *S.* Dublin and those which are published tend to take a different approach to the one described here. Specific loci or regions of interest appear to be sought and reported upon and rarely include detail about genes associated with fimbriae, flagella or nutrient acquisition (García-Soto et al., 2021; Klose et al., 2022; Langridge et al., 2015; Mohammed and Cormican, 2016; Mohammed et al., 2017). Therefore, comparison of these genetic elements from the isolates in this study to those in the literature is not possible.

It is likely that both SPI-6 and SPI-19 were present in the *S.* Dublin isolates, as has been previously reported (Blondel et al., 2009; Langridge et al., 2015; Mohammed and Cormican, 2016; Mohammed et al., 2017). SPI-6 encodes shiga-like toxin A genes *sciR* and *sciS* (also known as *tssM*) and both genes were identified in the isolates (Mohammed and Cormican, 2016). Additionally, elements of the *saf* fimbrial operon were present in all of the isolates which is also encoded on SPI-6 (Blondel et al., 2009). SPI-19 includes an *impA* sequence which was identified in all of the isolates (Blondel et al., 2009). Therefore, the duplicates of *tssC, tssF, tssG, tssJ* and *tssK* as well as these SPI-specific genes could be indicative of the presence of both of these T6SS and SPIs. Previous reports have included that the vi-antigen coding region SPI-7 is rarely present in *S.* Dublin isolates, however the presence of both *tvi* and *vex* genes in this study in all isolates, all be it in incomplete operons, seems to contradict this (Klose et al., 2022; Mohammed et al., 2017).

Gene clusters and operons which have not been previously described in *S.* Dublin include those associated with nutrient acquisition. It is unsurprising that the magnesium transport operon *mgtCB* was

identified in all of the isolates as this gene allows for bacterial survival intracellularly, an essential part of the pathogenesis of bovine abortion (Smith et al., 1998). Similarly, the complete operon *iroBCDEN* and its regulator *fur* are associated with acquisition of Fe^{3+} intracellularly (Leon-Sicairos et al., 2015).

Complete curli fibre operons (*csgAB* and *csgDEF*) were not identified in any of the 16 isolates, implying that curli biogenesis could be attenuated in these strains. Curli fibres are potent activators of host immune responses, so *S.* Dublin isolates which are unable to express curli fibres may be more likely to cause invasive disease (Tükel et al., 2010). It has been suggested that the loss of curli could be advantageous as this is associated with a more virulent phenotype in *S.* Typhimurium (Ahmad et al., 2011). The loss of different outer membrane proteins like curli fibres is also associated with the adaptation to particular hosts, potentially demonstrating the evolutionary adaptations of *S.* Dublin towards bovine hosts (Mackenzie et al., 2019).

Most unanticipated was that the VFDB implied that flagellar genes *fliC* and *fliD* were not present in any isolates in this study. Repression of *fliC* expression has been described as a mechanism of host immune evasion, as the immune response is dampened when *fliC* is not expressed (Ogushi et al., 2001; Yim et al., 2014). However, *fliC* mutation and repression of expression significantly attenuates invasion of human embryonic intestinal cells (INT-407) so it is unlikely that the *S.* Dublin isolates in this study were not able to express functional flagella (Olsen et al., 2013). *fliC* is a highly studied gene in *S.* Gallinarum, as its pseudogenisation has accompanied host adaptation in this serovar (Li et al., 1993). Inter-serovar recombination of *fliC* has been reported in *S.* Typhi, where the *fliC* gene differs substantially between isolates of different lineages in the same serovar (Frankel et al., 1989). The sequence of *fliC* in *S.* Enteritidis and *S.* Dublin differ by three nonsynonymous substitutions, demonstrating the divergence between serovars (Selander et al.,

1992). DNA sequences encoding *fliC* in *S.* Dublin and *S.* Typhimurium are reportedly only 38% similar and others have reported difficulties identifying *S.* Dublin *fliC* using databases which do not include a serovar specific reference (Olsen et al., 2013; Reen et al., 2005). Upon further investigation, the VFDB has record only of *fliC* in *S.* Typhimurium LT2, so it is likely that *fliC* was not identified because of this (Liu et al., 2017). This again demonstrates that the use of the VFDB may be limited when investigating under-researched serovars like *S.* Dublin of which there are no or few annotated genomes to inform such a database.

Another limitation in comparing whole genome sequences to databases such as the VFDB is that the results can be influenced by the quality and coverage of the whole genome sequences used. Whilst there was very little variation observed between these isolates, it is possible that the variation that was observed could have been due to issues with the quality of the constructed genome alignments. At the time of writing, there has not been a study of the validity of the use of the VFDB. One way of validating its use could be to use a large cohort of different well-annotated *Salmonella* genomes and compare the outputs of the VFDB to that already known of the genomes. It is also unclear as to how the algorithm in the VFDB functions, so the presence/absence studies are presumptive and require further verification using both bioinformatic and wet-laboratory approaches. These approaches have been discussed in a later section, 3.4.1 - Limitations of the Virulence Factor Database Approach.

2.5 - Summary

As would be expected, all 16 isolates evaluated in this chapter were similar across the panel of different characterisation methods. The few differences which were identified could be considered in future experiments, where they could be excluded or further investigated. However, as there is relatively little in the literature about the virulence factors of *S.* Dublin, it was difficult to compare and contrast the findings of this study to others findings. Additionally, it was not

possible to determine whether these isolates were truly representative of the population of *S.* Dublin isolates in the UK in terms of the presence or absence of particular virulence factors. Comparison of these isolates to those of a larger population of UK isolates from various origins of isolation could overcome this. Additionally, comparison of *S.* Dublin isolates with the more frequently studied and better characterised *S.* Typhimurium isolates could aid in understanding core virulence factors associated with infection of humans or livestock.

Chapter 3 - Computational analysis of *Salmonella* Dublin and Typhimurium isolates from various origins

3.1 - Introduction

As previously stated, many studies using *S.* Dublin use smaller subsets of isolates, or specific isolates like 2229 and 3246. Whilst this can be a useful baseline for wild-type isolates in mutation studies, it is possible that these isolates may not be representative of the currently circulating population, as has previously been demonstrated with isolate 2229 differing from 15 UK bovine abortion associated isolates. Since all of the isolates studied here thus far have been isolated from cattle, it is also possible that adaptations for particular hosts have not yet been observed, as *S.* Dublin isolates from humans have not been analysed. The process of host adaptation involves the acquisition of genetic elements conveying advantages for particular niches and degradation or pseudogenisation of redundant sequences (Langridge et al., 2015). The presence of specific virulence loci is associated with host adaptation and this evolutionary process and can be mapped based on the genetic relatedness of different serovars (Baumler et al., 1998). An example of this pseudogenisation and host adaptation is that of the gradual loss of flagellar gene *fliC* resulting in the loss of motility in *S.* Gallinarum, a host-restricted serovar which infects chickens (Li et al., 1993). Therefore, it is important to analyse a range of different isolates from a range of different origins of isolation in order to discern if there are host-specific adaptations which could ultimately lead to the evolution of host restriction.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a host-generalist serovar related distantly to *S.* Dublin in comparison to *S.*

Enteritidis or *S.* Choleraesuis (Baumler et al., 1998). *S.* Typhimurium has a broader host range compared to *S.* Dublin, infecting pigs, poultry, and rodents as well as humans and livestock. *S.* Typhimurium infects both livestock and humans, with disease varying in severity from self-limiting diarrhoea to severe systemic infection (APHA, 2019b; Public Health England, 2018). *S.* Typhimurium infection of pregnant cattle rarely causes abortion, but such cases have been identified in the UK (APHA, 2021a). Due to its history as a murine of human *S.* Typhi infection, much more is known about virulence in *S.* Typhimurium and molecular mechanisms behind pathogenesis compared to *S.* Dublin. Therefore, it may be possible to compare *S.* Dublin and *S.* Typhimurium virulence factors and identify any livestock or serovar-specific virulence factors of interest which could aid in explaining the mechanisms behind abortions in cattle.

3.1.1 - Aims and hypotheses

This chapter aims to characterise a larger group of *S.* Dublin isolates from the UK from various origins of isolation and years to discern whether the 16 isolates previously used are representative of a wider population. Furthermore, comparison of the virulence genes of *S.* Dublin isolates from the UK with those from *S.* Typhimurium isolates also from the UK will aid in identifying any host-specific and cross-serovar virulence genes associated with disease in particular species.

We hypothesise that there may be inter-serovar variation in the presence or absence of virulence genes in *S.* Dublin and *S.* Typhimurium isolates due to their different host specificities. Additionally, there may be intra-serovar differences based on the origin of isolation.

3.2 - Methods

The whole genome sequences of 250 *S.* Dublin isolates from the UK, isolated between 2001 and 2019 were downloaded from Enterobase on the 28th October 2019 (Achtman et al., 2020; Alikhan et al., 2018). The criteria for isolate selection were that the isolates were listed as being "*Salmonella enterica* serovar Dublin" and that there was information about the isolates origin of isolation and the year of isolation in the accompanying metadata (Appendix Table 9.4). The origins of isolation included "bovine" (n=74), "canine" (n=4), "environment" (n=2), "food" (n=21), "human" (n=128), "livestock" (n=12), "other mammal" (n=2), "ovine" (n=4), "retail meat" (n=1) and "wild animal" (n=2). These isolates were analysed alongside the 15 bovine abortion isolates from the UK in 2017 and calf diarrhoea reference isolate 2229 to gain a better understanding of how these isolates compared to a wider population. In total, 266 *S.* Dublin isolates were included in these analyses. To simplify these analyses, different origins of isolation were grouped into "livestock" (including "bovine", "livestock", "ovine", "bovine abortion" and "calf diarrhoea", n=106), "human" (n=128), "food" (n=21), and "other" (including "canine", "environment", "other mammal" and "wild animal", and "retail meat", n=11).

A cohort of 266 *S.* Typhimurium isolates were selected from Enterobase and downloaded on 21st May 2021. Selection was based on the origin of isolation and, as much as possible, the year of isolation to mirror the metadata of the *S.* Dublin isolates. This therefore included "livestock" (n=106), "human" (n=128), "food" (n=21) and "other" (n=11). It was not always possible to match the dates of the *S.* Typhimurium isolates to the *S.* Dublin isolates, so this cohort had a larger range of years of isolation (1994 to 2020).

3.2.1 - Core and Accessory Phylogenetic Alignment and MLST of *S.* Dublin isolates

Multilocous Sequence Typing (MLST) analysis was performed by Dr Adam Blanchard (University of Nottingham) using ChewBBACA based

on a 95% loci presence (Silva et al., 2018). Core and accessory genome alignment was completed using Roary by Dr Adam Blanchard, and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015). The core genome was defined as the collection of genes present in every isolate in the study, whilst the accessory genome was identified as being any gene which showed variation within the isolates.

3.2.2 - Identification of virulence factors in *S.* Dublin and *S.* Typhimurium isolates

The identification of virulence factors in both *S.* Dublin and *S.* Typhimurium isolates was carried out as described in Chapter 3.

The *S.* Typhimurium virulence gene data cleansing, including functional categorisation of virulence genes which were not identified in *S.* Dublin isolates, was completed by second year Animal Science student Sophia Lewin under the direction and supervision of Jemma Franklin.

3.2.3 - Statistical Analysis

A Fisher's Exact Test was used to discern the likelihood of finding a virulence factor in isolates found more often in livestock compared to human isolates.

3.3 - Results

Characterising a larger group of *S.* Dublin isolates should give an insight into whether the 16 isolates previously used are representative of a wider population in the UK. Additionally, comparing the virulence of isolates of the well-characterised serovar *S.* Typhimurium may aid in understanding *S.* Dublin virulence. Identifying differences between origins of isolation could also provide an insight into species or host-specific virulence factors.

3.3.1 - Phylogenetic relationships between *S.* Dublin isolates

Phylogenetic alignment of all 266 *S.* Dublin isolates based on core genome did not reveal distinct clustering with relation to origin of isolation due to their high level of similarity (Figure 3.1). Alignment of these isolates based on their accessory genomes resulted in distinct clustering of "livestock" associated isolates separate to "human" and "food" associated isolates (Figure 3.2).

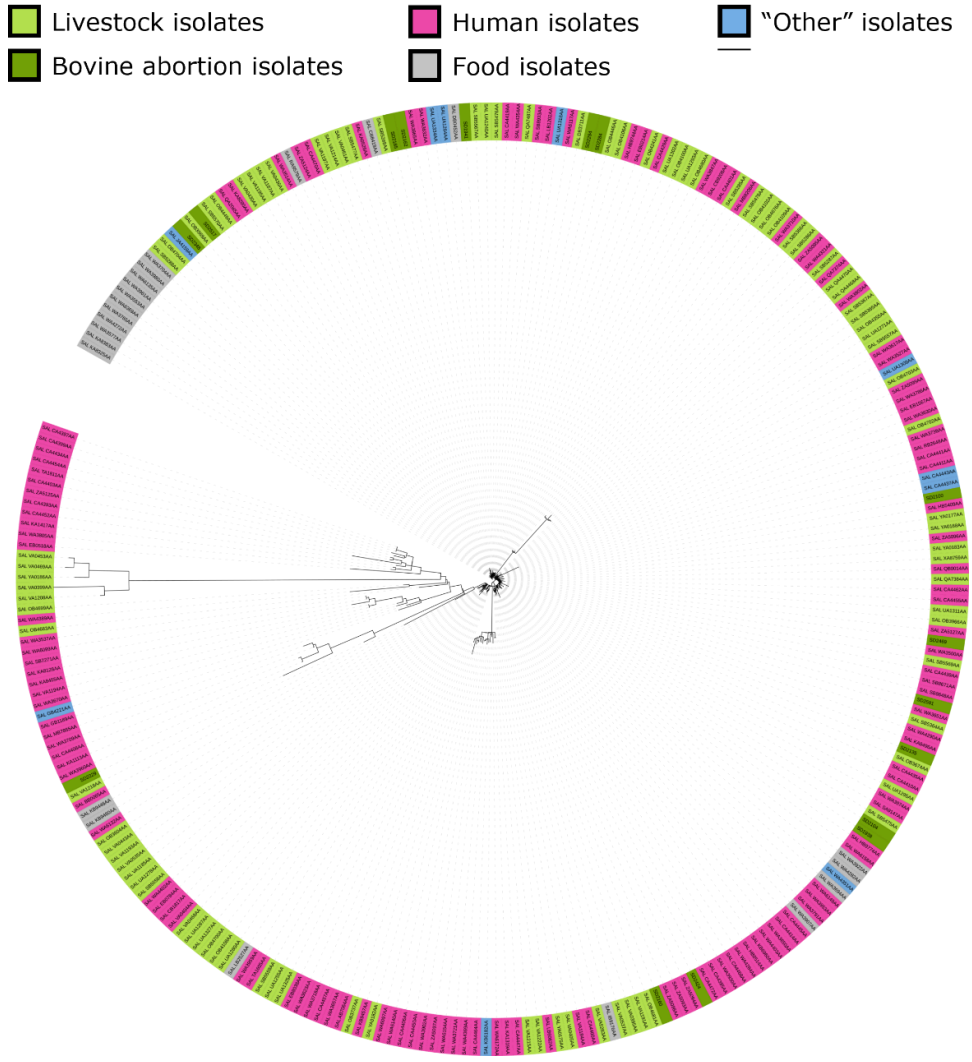


Figure 3.1 – Phylogenetic alignment of the core genomes of 266 *S.* Dublin isolates from various origins of isolation. Phylogenetic alignment based on the core genome was completed by Dr Adam Blanchard (University of Nottingham, United Kingdom) using Roary and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015). Core genome was defined as being any genes which were ubiquitous among all of the isolates in the study. Origins of isolation were livestock (green), humans (pink), food (grey) and "other" (blue). Isolates from cases of bovine abortion in the UK in 2017 are indicated in dark green.

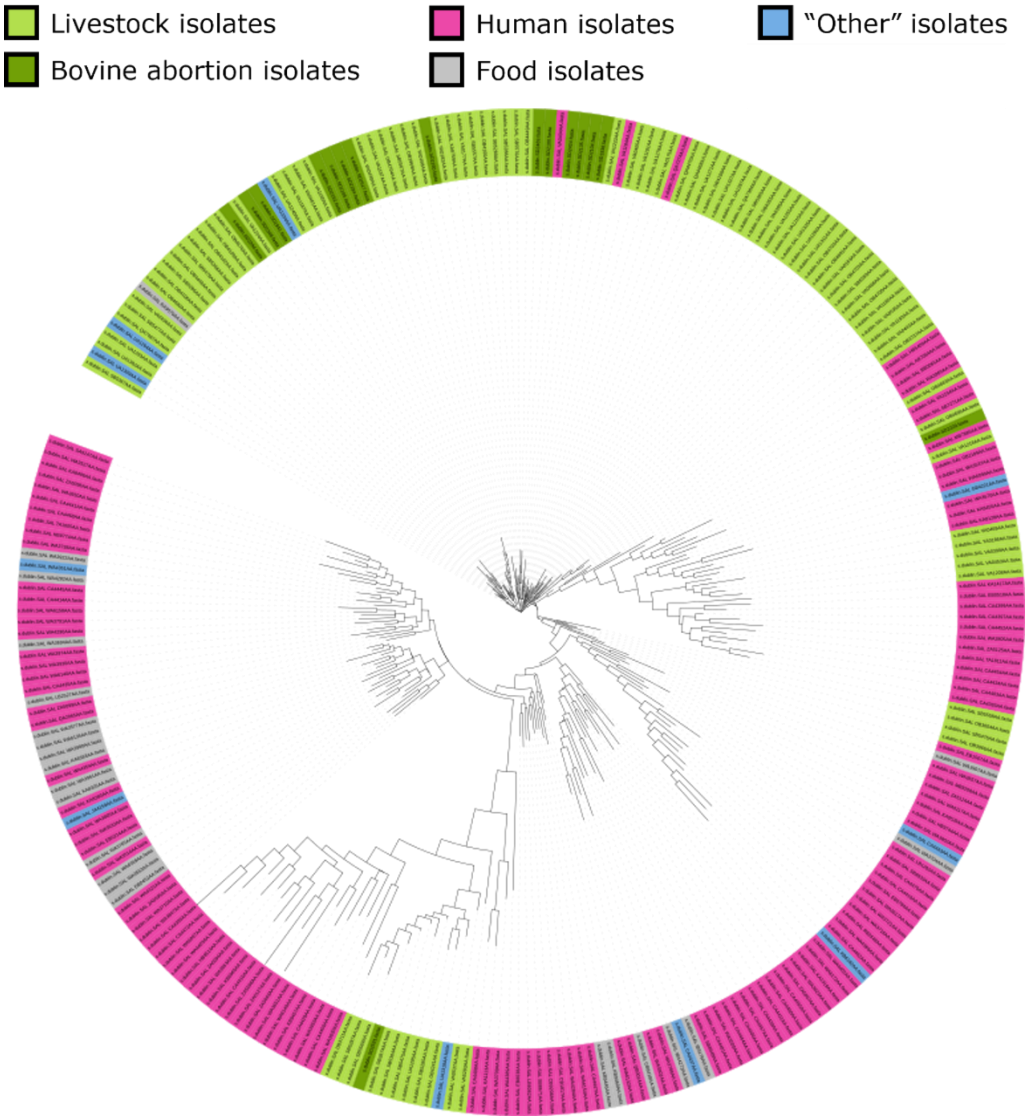


Figure 3.2 – Phylogenetic alignment of the accessory genomes of 266 *S.* Dublin isolates from various origins of isolation. Phylogenetic alignment based on the core genome was completed by Dr Adam Blanchard (University of Nottingham, United Kingdom) using Roary and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015). The accessory genome was defined as any gene which was not present in all of the isolates in the study. Origins of isolation were livestock (green), humans (pink), food (grey) and "other" (blue). Isolates from cases of bovine abortion in the UK in 2017 are indicated in dark green.

3.3.2 - *S.* Dublin Virulence Factors

The following "VFDB-inferred" results may reflect some of the limitations of using the VFDB approach to identifying virulence factors, rather than showing the true presence/absence of particular virulence genes.

The evolution of *Salmonella* serovars appears to allow the development of tropism-specific virulence profiles which ultimately leads to the appearance of host-restricted and host-adapted serovars like *S.* Dublin (Rakov et al., 2019). Therefore, investigating different virulence factors in a wider population may reveal specific changes in the virulence profiles which could be more often associated with infection in particular species. Furthermore, comparison of the previously characterised isolates with a larger UK population allows understanding of how representative those isolates are of a wider population with respect to their virulence factors.

A total of 285 virulence genes were identified in the 266 isolates used in this analysis, an additional 41 genes compared to the previous study using only 16 isolates (Figure 3.11). Virulence genes were again categorised into 20 different functional groups and two additional groups identified in the 16 isolates previously used. These groups comprised one gene each – SPI1 (*sprB)* and sensor (*phoQ*). Genes associated with SPI1 (*invC, sopE2* and *spvC*), SPI2 (*pipB, pipB2, ssaC, sseG*), T6SS (including second copies of *tssB* and another *hcp* gene as well a single copy of *vgrG*), curli fibres (*csgD* and *csgF*), fimbriae (*bcfB, pegC, steD, stfD, stfF, stiA*, *sthB* and *safD*), vi antigen (*tviB, tviC, vexA, vexC, vexD* and *vexE*) mobile genetic elements (*pilM, pilN, pilO, pilP, pilQ, pilR, pilS, pilT, pilU, pilV, pilV2* and *pilK*) and an additional hypothetical or discontinued gene *(sciJ*) were identified in this larger cohort of *S.* Dublin isolates (Figure 3.11).

The 266 isolates were grouped into 46 virulence profiles. VP1 and VP19 represented 110 (41.35%) and 88 (33.08) isolates respectively whilst only differing by the presence or absence of SPI2 gene *ssaU* (Figure 3.4). Ten virulence profiles represented more than one isolate (VP20 n=7; VPs 2 and 21 n=5; VP22 n=4; VP23 n=3; VPs 3, 4, 24, 25 and 26 n=2) and the remaining 34 virulence profiles represented one isolate each (Figure 3.12). Livestock isolates were predominantly represented by VP1 (86 isolates of 106) and the remaining 20 isolates were represented by 15 VPs (Figure 3.12). Human isolates were

predominantly represented by VP19 (61 isolates of 128) and the remaining 67 isolates were represented by 32 VPs (Figure 3.12). The 22 food VPs were split into VPs 1 and 19 (5 and 17 isolates respectively) and the 10 "other" isolates were represented by 4 VPs (Figure 3.12). These 46 virulence profiles arose due to variation in the presence or absence of 87 of the 285 genes.

In total, *ssaU* with ≥90% sequence homology was identified in exactly half (n=133) of the isolates evaluated. Livestock isolates (96 out of 108) were statistically more likely to have *ssaU* with a ≥90% sequence homology compared to human isolates (27 out of 128) when challenged in a Fishers Exact Test (P=<0.0001). 129 isolates were >80% but <90% homologous and three human isolates were less than 80% identical (79.88%, 69.14% and 43.55% sequence identity). Isolate L 2160/17 was the only one isolate to have no sequence homology for *ssaU* at all, as described in the previous chapter. The importance of *ssaU* in the functionality of the SPI2 T3SS implies that it is highly unlikely that this gene would be "absent" in virulent isolates (Yu et al., 2018).

Five isolates associated with livestock and represented by five different virulence profiles (VP5, VP6, VP7, VP8 and VP27) were the only isolates harbouring a total of 28 different virulence genes. T3SS SPI1 genes *invC, sopE2* and *spvC* were present in all five isolates, and they differed further in that *invJ* and *sopD2* were not present in these isolates (Figure 3.3). Vi antigen genes *tviBCDE* and *vexABCDE* were only identified in these five isolates (Figure 3.9), along with two copies of T6SS gene *tssB* and three copies of T6SS gene *hcp* and T6SS gene *vgrG* (Figure 3.5). Fimbrial genes also differed in these isolates, where *bcfB, stfD, safD* and *sthB* were present, whilst *bcfA, fimF* and *sthC* were not (Figure 3.6). A *fimW* sequence with sufficient homology was also absent in these isolates but this was a common difference between virulence profiles, with 24 of the 46 virulence profiles having this difference (Figure 3.6). Mobile genetic element genes *pilMNOPQRSTUVV2K* were present in four of these five isolates (Figure

3.10). The five isolates all clustered closely in the accessory phylogenetic alignments (Figure 3.2).

The most variation between VPs was observed in the fimbrial gene category, which arose due to the presence of absence of 22 of the 56 fimbrial genes identified (Figure 3.6). As observed in the 16 isolates previously characterised, the only "intact" fimbrial gene clusters were *lpf* and *sti*. Adhesin *bcfA* was absent in the five livestock isolates previously described, but all other adhesins previously identified in the 16 isolates (*bcfA, fimH, sthE, stiH, sefD, pegD, safD*) were present in all other isolates (Figure 3.6). Similar to the previous characterisation of the 16 isolates, the adhesins from other fimbrial gene clusters previously described to be absent were also absent in all other isolates (*stdD, steG, stfH* and *pefD*). Previous studies have characterised many more fimbrial clusters in *S.* Dublin isolates, so it is unlikely that these findings are correct (Yue et al., 2012).

The same chemotaxis, magnesium uptake, T4SS, adherence, AMR, DUF, DcrB-related, toxin and "no record" genes were identified in all of the isolates. The flagella, SPI1/2, "hypothetical or discontinued" and sensor gene categories were almost identical across all of the isolates, where the difference in the presence or absence of these genes in a single isolate gave rise to additional virulence profiles (represented by VPs 11, 39, 28 and 20 respectively, data not shown). The ferric uptake gene category was largely identical across all isolates, apart from two VPs, one in which *fur* could not be identified, and another in which the *iroBCDEN* operon could not be identified (Figure 3.8). Iron uptake genes which would have been assumed to be present (including the *fep* and *ent* operons) were not identified, indicating that these may be missing from the VFDB (Nagy et al., 2013). Similarly, the outer membrane protein functional category was largely similar, but *ompD* was not present in four isolates and both *ompD* or *apE* were not present in one other isolate.

Six genes were identified in individual isolates, which included SPI2 gene *pipB*, (Figure 3.4) curli genes *csgD* and *csgF*, (Figure 3.7)

fimbrial genes *pegC* and *steD* (Figure 3.6) and sensor gene *phoQ* (represented by VPs 37, 5, 24, 32 (*pegC* and *steD*) and 20 respectively). As some of the results presented here do not make biological sense, all of the results should be verified using an alternative bioinformatic method before being considered an accurate reflection of the presence/absence of virulence factors in *S.* Dublin isolates from the UK.

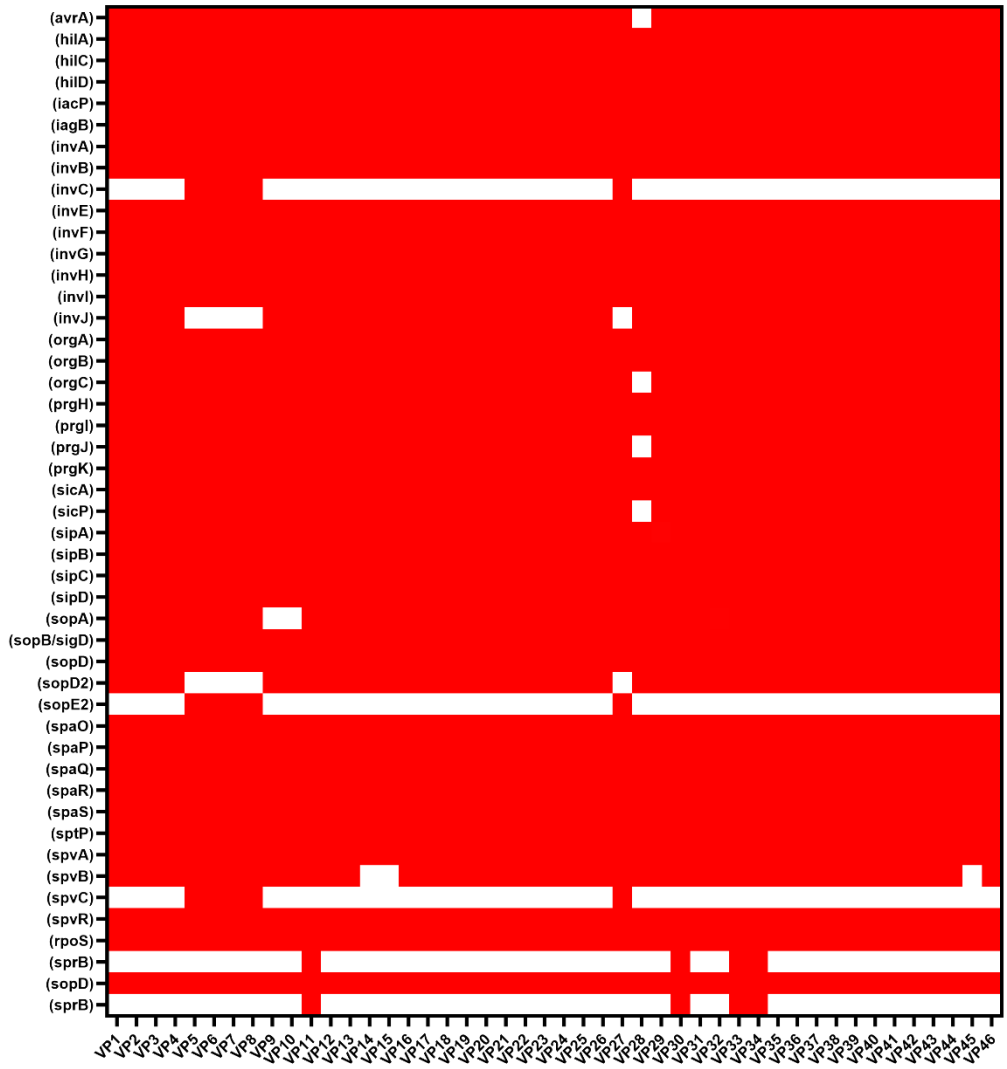


Figure 3.3 – VFDB-inferred presence and absence of SPI1 genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

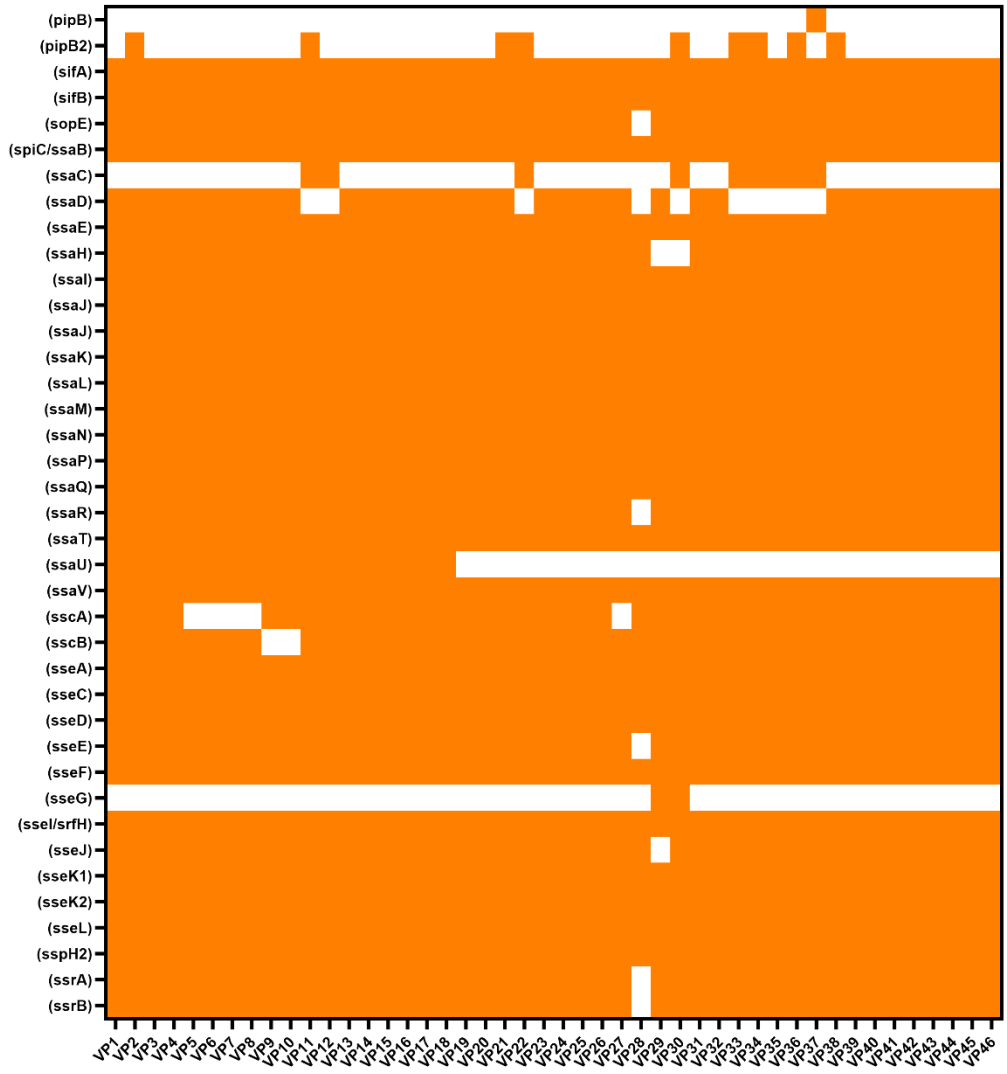


Figure 3.4 - VFDB-inferred presence and absence of SPI2 genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

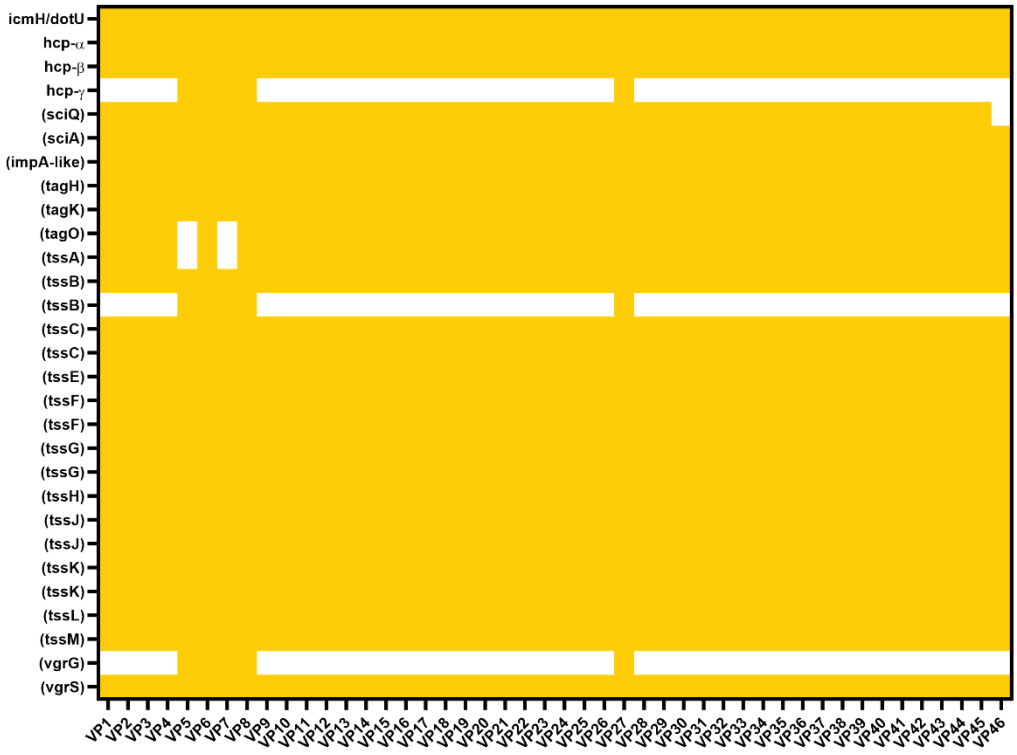


Figure 3.5 - VFDB-inferred presence and absence of T6SS genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

Figure 3.6 - VFDB-inferred presence and absence of Fimbrial genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

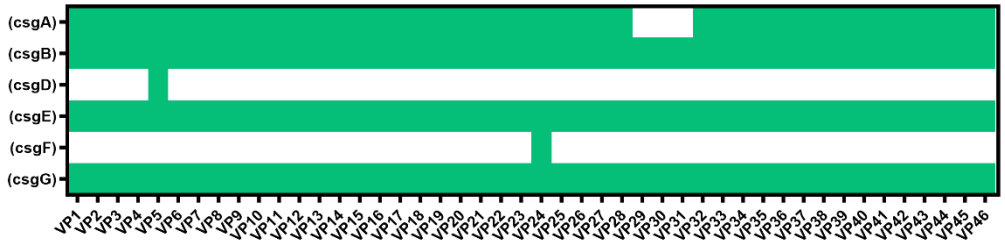


Figure 3.7 - VFDB-inferred presence and absence of curlin-associated genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

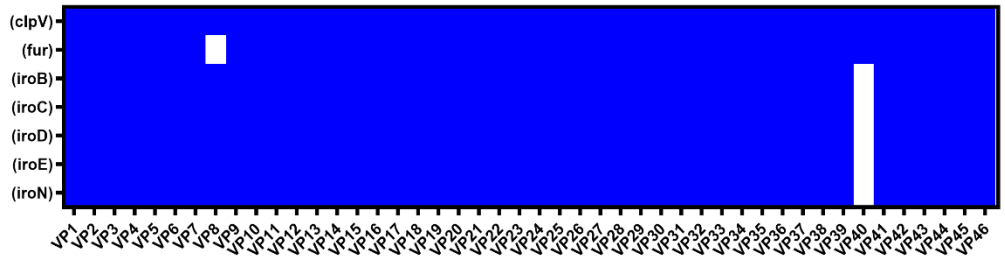


Figure 3.8 - VFDB-inferred presence and absence of ferric-uptake genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

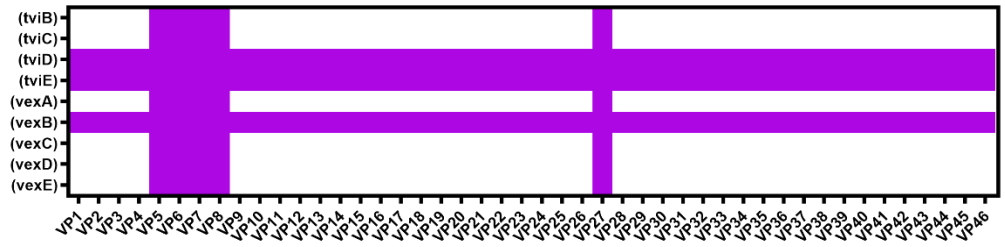


Figure 3.9 - VFDB-inferred presence and absence of vi antigen genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

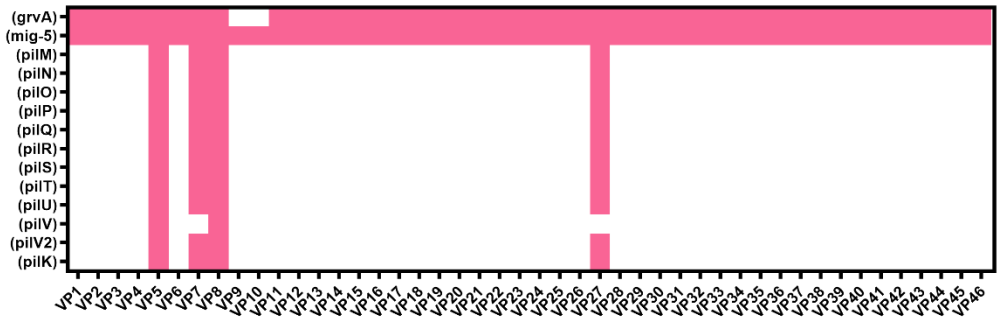


Figure 3.10 - VFDB-inferred presence and absence of mobile genetic element genes across 46 virulence profiles of 266 *S.* Dublin isolates. The whole genome sequence of 266 *S.* Dublin isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

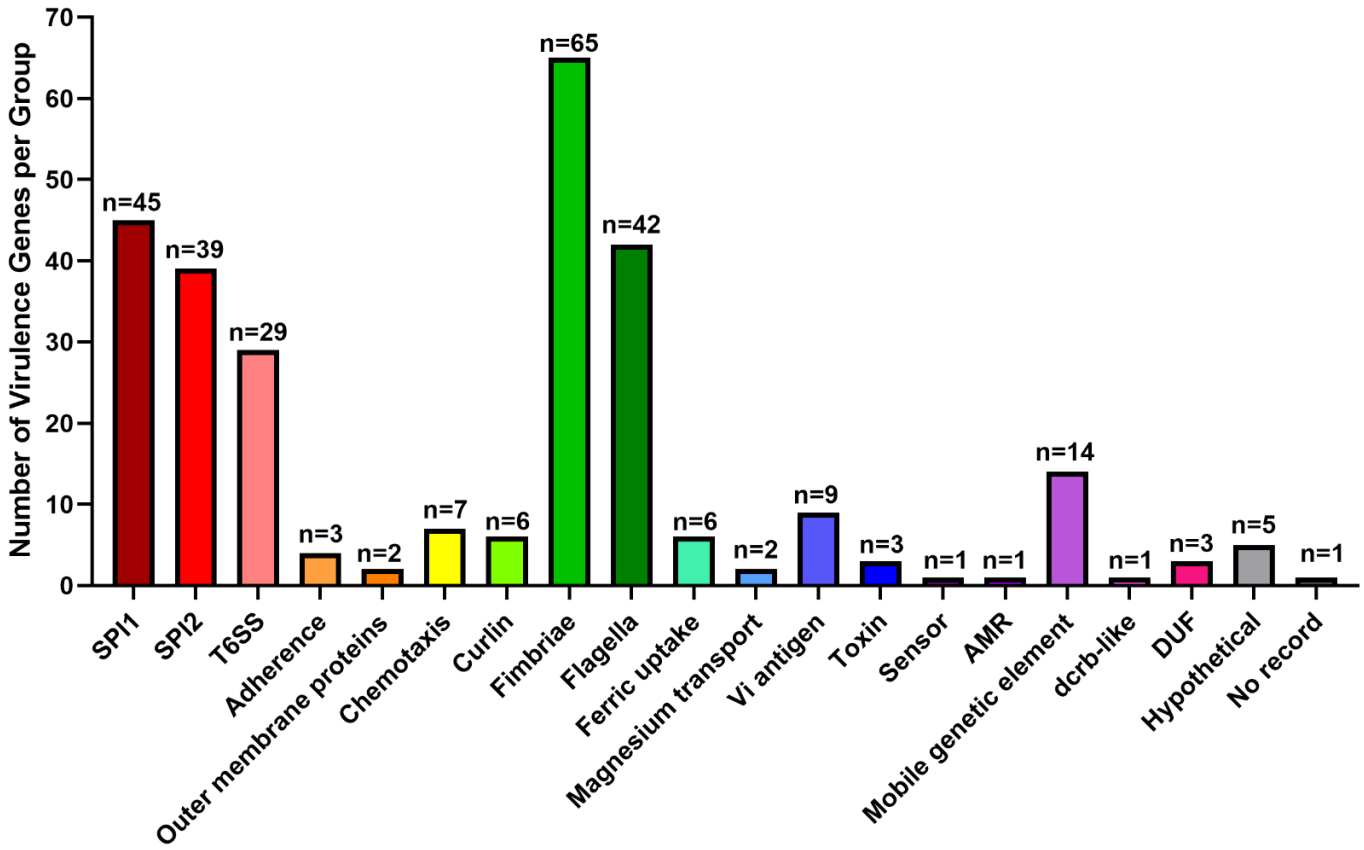


Figure 3.11 – Number of virulence factors within 20 functional categories identified in 266 *S.* Dublin isolates. The identification of virulence factors was completed using the VFDB (Virulence Factor Database, Bo Liu et al., 2019), where genes with >90% sequence homology were classed as being "present". Investigation of the function of each virulence factor was completed using NCBI Gene and NCBI Protein and genes were grouped based on these functions. The number of genes in each functional group is indicated. Total number of virulence factors (n) = 285.

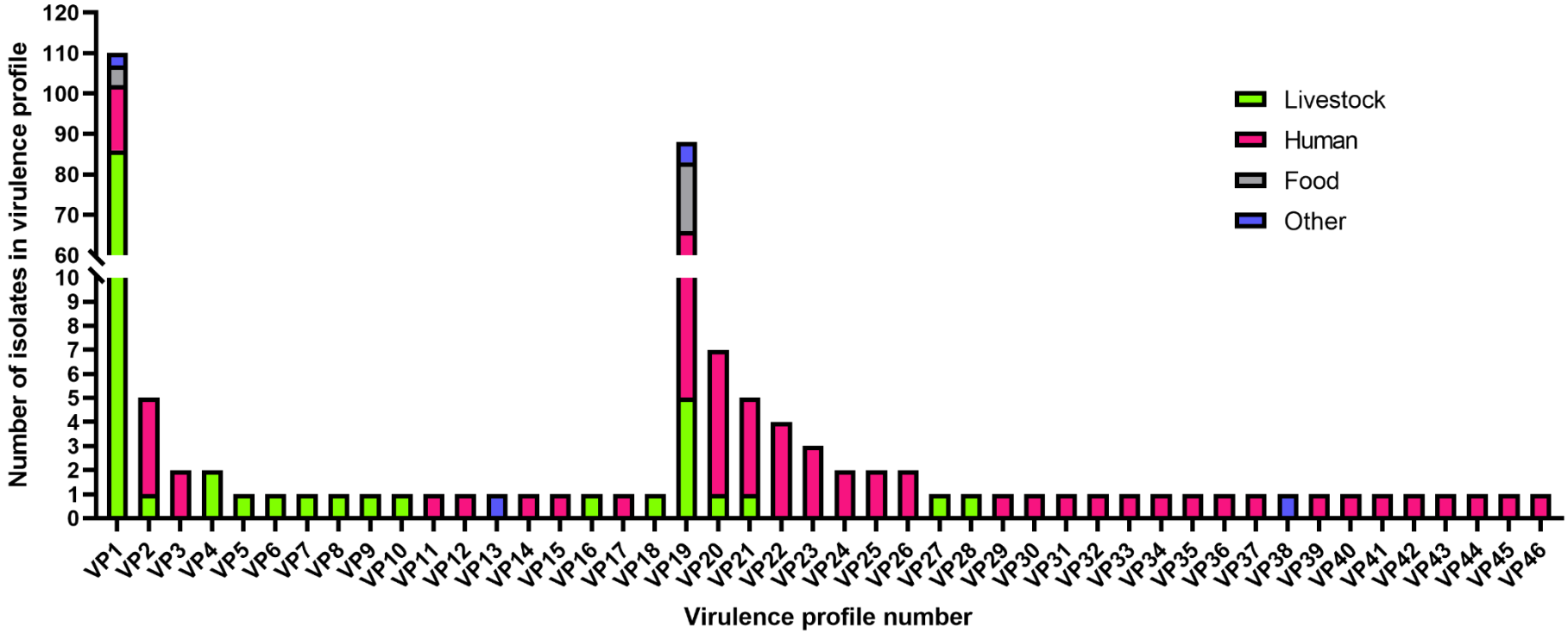


Figure 3.12 – Number and origin of *S.* Dublin isolates in each Virulence Profile (VP) divided into the isolates origin of isolation. Virulence profiles were determined by the VFDB-inferred (Virulence Factor Database, Bo Liu et al., 2019) "presence" (sequence homology of >90%) or "absence" (sequence homology <90%) of different virulence genes and are presented with their origins of isolation (green = livestock; pink = human; grey = food; blue = "other"). n=266

3.3.3 - *S.* Typhimurium Virulence Factors

S. Typhimurium has been studied extensively due to its use as a model in mice of human typhoid infection with *S.* Typhi. Therefore, there is a larger body of literature from which results can be interpreted for *S.* Typhimurium virulence compared to *S.* Dublin. By comparing *S.* Dublin isolates to *S.* Typhimurium isolates from similar origins of isolation, it may be possible to identify host-specific virulence factors which are conserved across different serovars capable of infecting the same hosts. However, understanding the virulence factors present in a cohort of *S.* Typhimurium isolates from origins of isolation similar to those of the *S.* Dublin isolates is necessary before the two serovars can be compared.

141 virulence genes were identified in 266 Typhimurium isolates which were categorised into 15 different functional groups. These groups included SPI1 T3SS (n=38), SPI2 (n=42), SPI3 (n=1), adherence (n=3), chemotaxis (n=2), curli (n=10), fimbriae (n=17), flagella (n=6), ferric uptake (n=18), magnesium transport (n=2), flippase (n=2), AMR (n=1), mobile genetic elements (n=4) and "hypothetical or discontinued" (n=2) (Figure 3.19). 32 genes were identified in the *S.* Typhimurium isolates which were not previously identified in the *S.* Dublin isolates, including one SPI1 gene (*slrP*), six SPI2 genes (*gogB, ssaG, ssaO, ssaS, sseB* and *sspH1*), one SPI3 gene (*misL*), one curli gene (*csgC*), fifteen ferric uptake genes (*entA, entB, entC, entE, entS, fepA, fepB, fepC, fepD, fepG, iucA, iucB, iucC, iucD* and *iutA*), three fimbrial genes (*pefA, pefC* and *pefD*), one flippase gene *(gtrB*), two mobile genetic elements (*sodC1* and *rck*), one outer membrane protein (*ompA*) and one hypothetical or discontinued protein (*nleC*). The SPI2 genes *sseK2* and *gogB,* mobile genetic element gene *rck* and flippase gene *gtrB* were present in two copies in some isolates (Figure 3.14, Figure 3.17 and Figure 3.18). Curli fibre genes *csgE, csgF* and *csgG* were present in two copies in only one isolate.

The 266 *S.* Typhimurium isolates were grouped into 86 unique virulence profiles. The largest number of genes in a single virulence profile was 136 (VP13) and the smallest number of genes in a single virulence profile was 116 (VP53) (Figure 3.20). VPs 1 to 9 were representative of 154 of the isolates, with the largest VP covering 26 isolates (Figure 3.20). These VPs differed in the presence or absence of SPI1 genes (*spvBCR*), SPI2 genes (*gogB, sseI/srfH, sspH2* and a second copy of *sseK2*), adherence genes (*shdA*), fimbrial genes (*pefABCD*), flippase genes (*gtrB*), and mobile genetic elements (*grvA* and *rck*) (Figure 3.13, Figure 3.14, Figure 3.15, Figure 3.17 and Figure 3.18). Human isolates were predominantly represented by VPs 1, 4, 5, 6, 7 and 8, whilst livestock isolates were predominantly represented by VPs 2, 3, 4, 5 and 9 (Figure 3.20). Of these genes, *spvBCR, pefABCD, grvA* and *rck* were significantly more likely to be associated with livestock isolates rather than human isolates ($P<0.0001$, Fishers Exact Test). *gogB* was also significantly more often identified in livestock isolates compared to human isolates ($P=0.0282$).

SPI2 was the most variable group across the different virulence profiles, where only seven genes were present ubiquitously (*sifA, sipC/ssaB, ssaC, ssaD, ssaS,* and *ssaT*) (Figure 3.14). Much of this variation was due to one livestock isolate in which sequence homology for *ssaE, ssaG, ssaH, ssaI, ssaJ, ssaK, ssaL, ssaM, ssaN, ssaO, ssaP, ssaQ, ssaV, sscA, sscB, sseA, sseB, sseC, sseD, sseE, sseF* or *sseG* was not identified (Figure 3.14). A non-homologous *ssaU* sequence with an identity of <90% (82.25%) was identified in one isolate of human origin (Figure 3.14). Due to the importance of these genes in the functionality of the SPI2 T3SS, it is unlikely that these genes would be "absent" in virulent strains (Yu et al., 2018).

In all isolates, the same magnesium transport (*mgtB* and *mgtC*), SPI1/2 (*spvB, spvC* and *spvR*), SPI3 (*misL*) and outer membrane protein (*ompA*) genes were identified (Figure 3.17 and Figure 3.18). The chemotactic gene *cheW* was less than 90% homologous in one

isolate, but both *cheW* and *cheY* were present in all other isolates. Similarly, single copies of curli genes *csgA, csgB, csgC, csgD, csgE, csgF* and *csgG* were present in all isolates, but two copies of *csgE, csgF* and *csgG* were identified in one isolate. The presence of ferric uptake genes was identical in all livestock-associated isolates (*entA, entB, entC, entE, entS, fepA, fepB, fepC, fepD, fepG, iroB, iroC,* and *iroN*) but homologous sequences were not present for *iroB, iroC* and *iroN* in five human isolates (Figure 3.16). Another human isolate was the only one to have homologous sequences for ferric uptake genes *iucA, iucB, iucC, iucD* and *iutA* (Figure 3.16). Functional iron uptake mechanisms are essential for virulence, so the findings that some of these genes are "absent" in presumably virulent strains may be incorrect (Nagy et al., 2013). There was little variation in the presence or absence of flagellar genes *flgG, flhC, fliA, fliG, fliM* and *flip,* other than one human isolate in which *flhC* could not be identified. The majority of structural, regulatory and accessory genes usually encoding the flagellar structure were not identified. Four fimbrial gene clusters were identified in all isolates which included γ-1 (*fim* and *lpf*), π (*ste*) and κ (*pef*) type fimbriae (Figure 3.15). Only the *lpf* operon was complete, but adhesins for all three operons were present (*fimH, lpfD* and *pefD*) (Figure 3.15). Previous work contradicts these findings, where a full complement of flagellar genes has been identified in *S.* Typhimurium isolates (Yue et al., 2012).

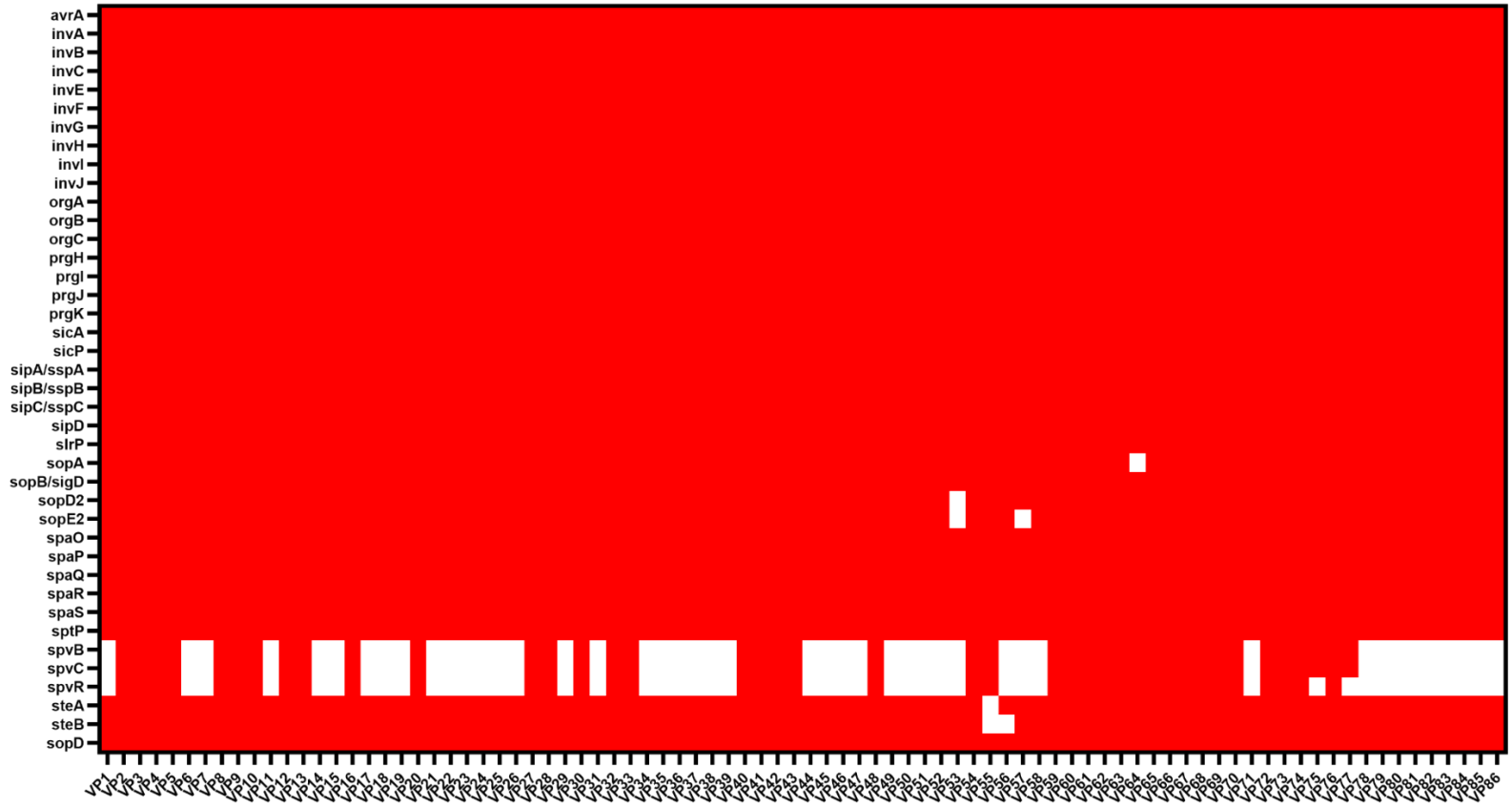


Figure 3.13 - VFDB-inferred presence and absence of SPI1 genes across 86 virulence profiles of 266 *S.* Typhimurium isolates. The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

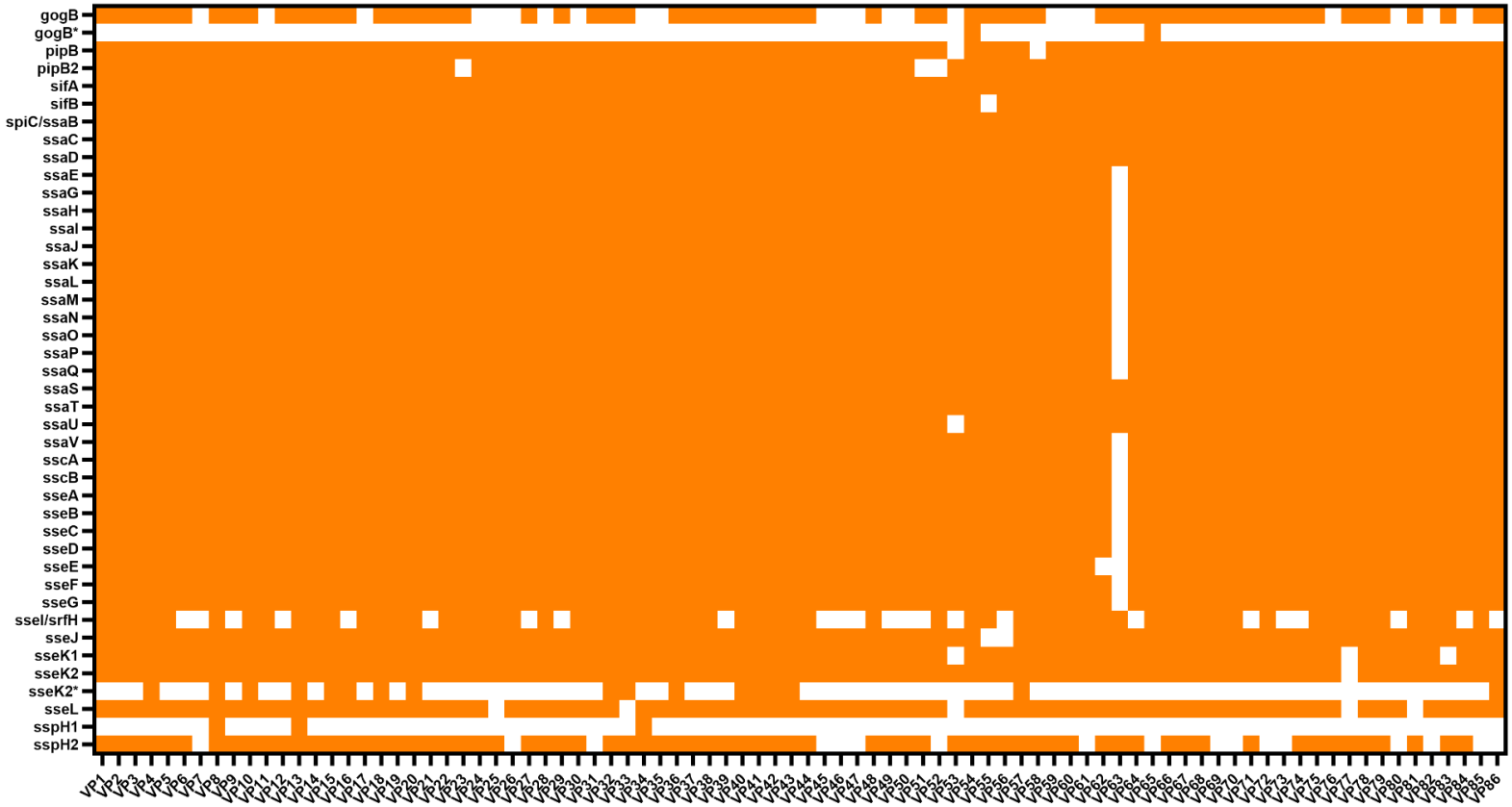


Figure 3.14 - VFDB-inferred presence and absence of SPI2 genes across 86 virulence profiles of 266 *S.* Typhimurium isolates. The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

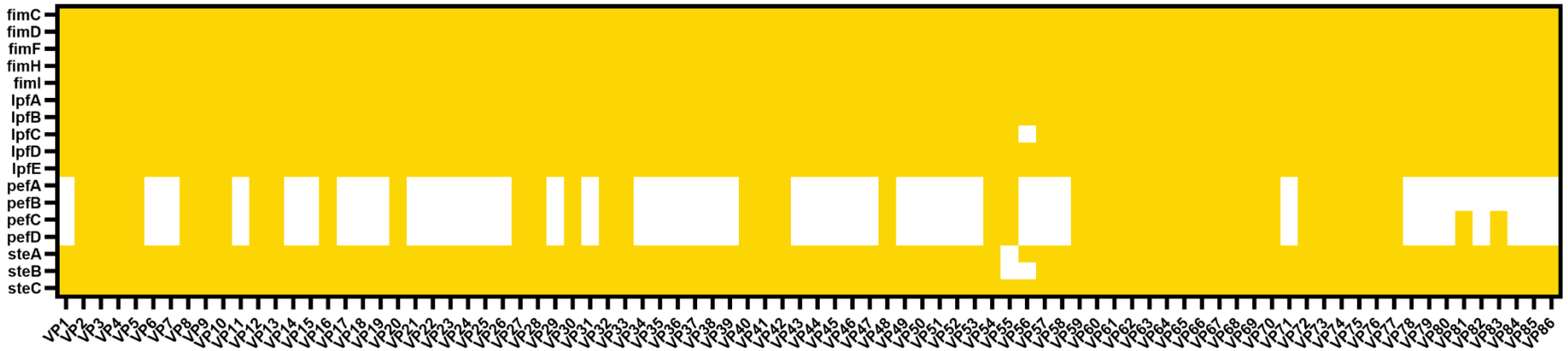


Figure 3.15 - VFDB-inferred presence and absence of Fimbrial genes across 86 virulence profiles of 266 *S.* Typhimurium isolates. The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

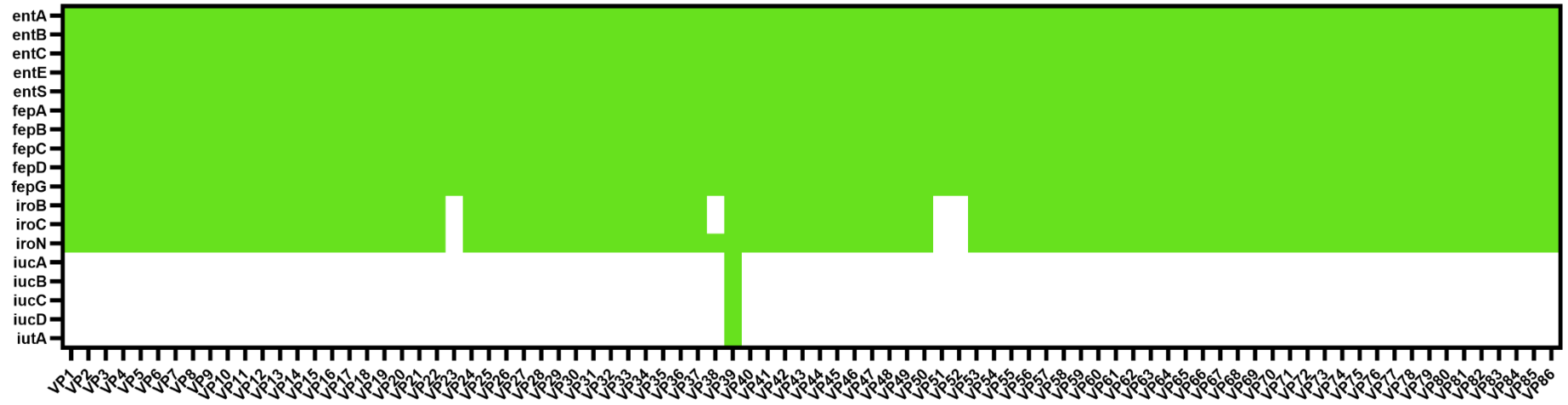


Figure 3.16 - VFDB-inferred presence and absence of ferric uptake genes across 86 virulence profiles of 266 *S.* Typhimurium isolates. The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB "absence" defined as there being <90% sequence homology.

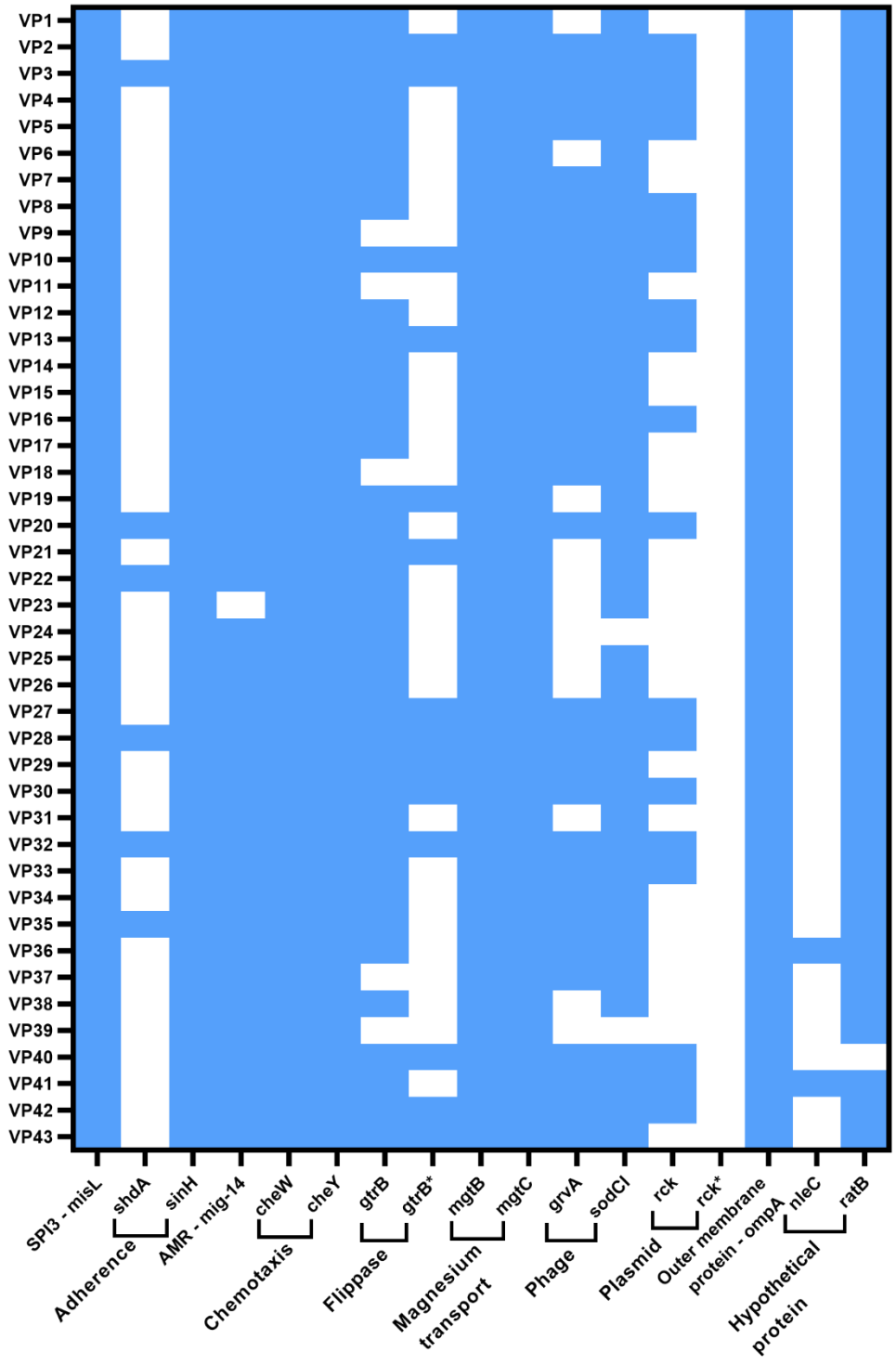


Figure 3.17 - VFDB-inferred presence and absence of "other" variable genes across 86 virulence profiles of 266 *S.* Typhimurium isolates (part A). The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB, "absence" defined as there being <90% sequence homology.

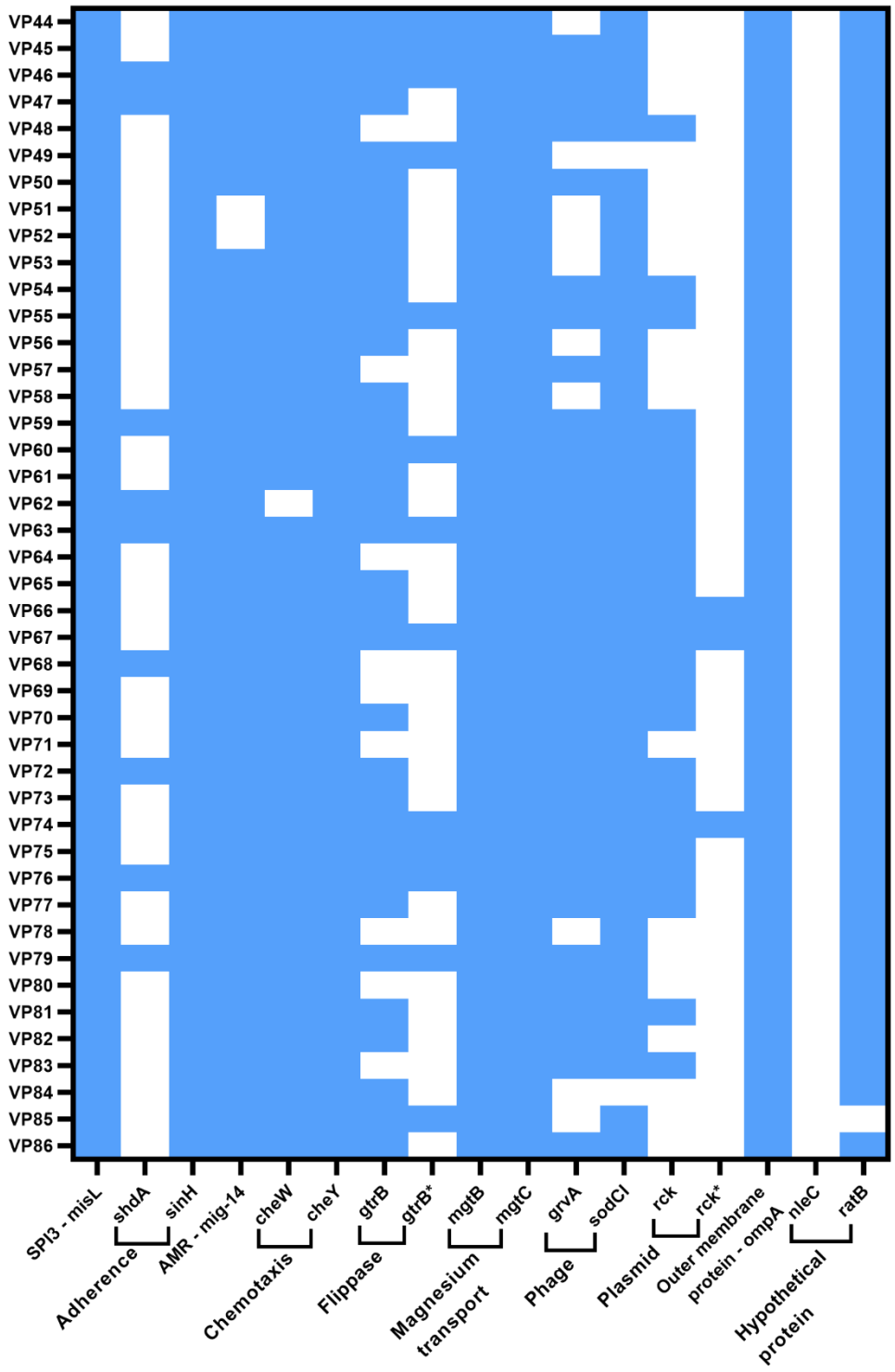


Figure 3.18 - VFDB-inferred presence and absence of "other" variable genes across 86 virulence profiles of 266 *S.* Typhimurium isolates (part B). The whole genome sequence of 266 *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). "Presence" of a gene is defined as the presence of sequence homology above 90% of a virulence gene according to the VFDB, "absence" defined as there being <90% sequence homology.

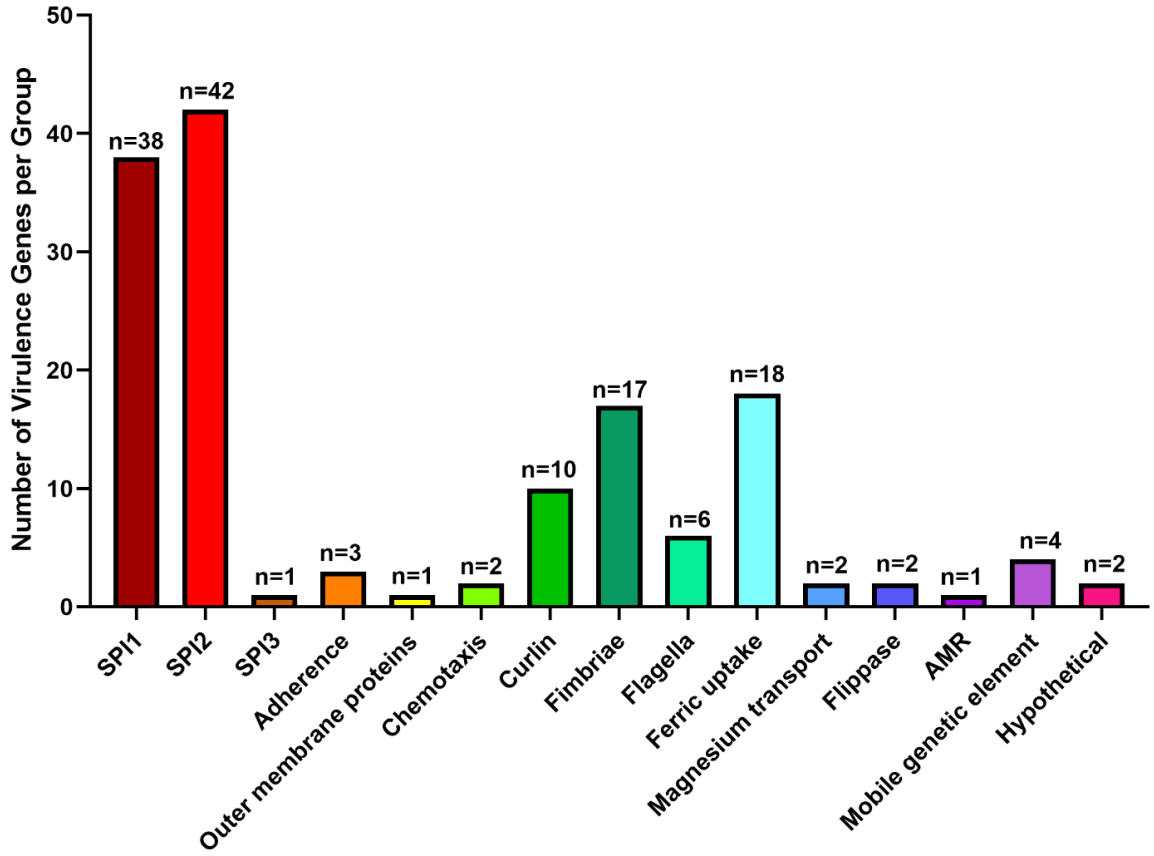


Figure 3.19 - Number of genes within the 15 functional categories identified in 266 *S.* Typhimurium isolates. The identification of virulence factors was completed using the VFDB (Virulence Factor Database, Bo Liu et al., 2019), where genes with >90% sequence homology were classed as being "present". Investigation of the function of each virulence factor was completed using NCBI Gene and NCBI Protein and genes were grouped based on these functions. The number of genes in each functional group is indicated. Total number of virulence factors (n) = 141

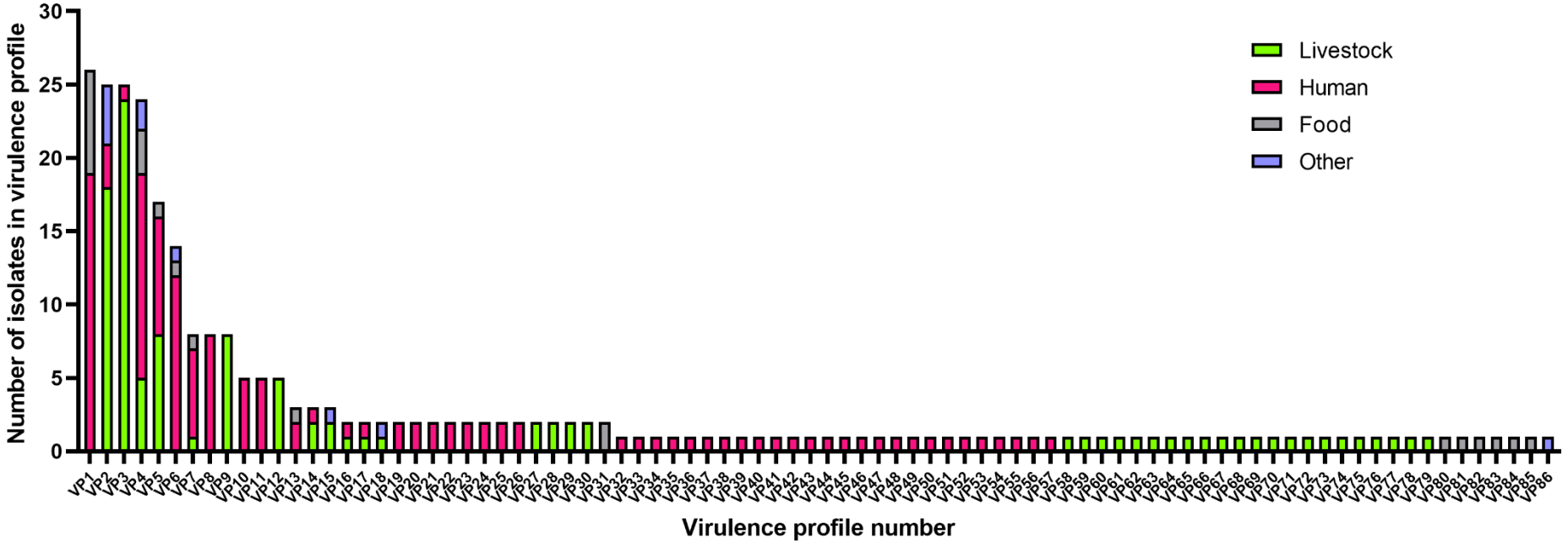


Figure 3.20 - Number and origin of *S.* Typhimurium isolates in each Virulence Profile (VP) divided into origins of isolation. Virulence profiles were determined by the VFDB-inferred (Virulence Factor Database, Bo Liu et al., 2019) "presence" (sequence homology >90%) or "absence" (sequence homology <90%) of different virulence genes and are presented with their origins of isolation (green = livestock; pink = human; grey = food; blue = "other"). n=266

3.3.4 - Comparison of *S.* Dublin and *S.* Typhimurium virulence factors

Identifying conserved genes across serovars and potentially across different origins of isolation could aid in understanding differences in host specificity.

More than twice the number of virulence factors were identified in *S.* Dublin isolates compared to *S.* Typhimurium (n=285 and n=141 respectively). The number of *S.* Typhimurium virulence genes in each isolate was distributed across a larger range compared to those in *S.* Dublin. Irrespective of serovar, there was a significant difference in the number of virulence genes in livestock isolates compared to human and food isolates ($P<0.05$) (Figure 3.22).

Genes identified in *S.* Dublin isolates were split into a total of 20 functional categories, whilst *S.* Typhimurium genes were split into only 14 (Figure 3.21). SPI3 gene *misL* and flippase gene *gtrB* were only present in *S.* Typhimurium isolates, whilst gene categories T6SS, toxin, vi antigen, sensor, "dcrb-like" and "DUF" were only present in *S.* Dublin isolates (Figure 3.21). Other than the SPI2, curli and ferric uptake categories, the number of virulence genes in each functional category was greater in *S.* Dublin isolates compared to *S.* Typhimurium (Figure 3.21). The magnesium transport (genes *mgtB* and *mgtC*) and AMR (gene *mig-14*) functional categories were identical in all isolates in both serovars (Figure 3.21).

45 T3SS SPI1 genes were identified in *S.* Dublin whilst only 38 genes were identified in *S.* Typhimurium isolates (Figure 3.21). T3SS SPI1 genes *hilA, hilC, hilD, iacP, iagB, spvA* and *rpoS* were present in *S.* Dublin isolates but not *S*. Typhimurium isolates, whilst in the same functional category *slrP* was not identified in *S.* Dublin isolates (Figure 3.21). This "VFDB-inferred" finding is inconsistent with the literature which shows that the SPI1 T3SS is highly conserved and functional in a range of isolates in different *Salmonella* serovars (Cui et al., 2021; dos Santos et al., 2021; Yan et al., 2022). In contrast, 42 T3SS SPI2

genes were identified in *S.* Typhimurium whilst 39 were identified in *S.* Dublin (Figure 3.21). *S.* Typhimurium isolates had sequences homologous with *ssaG, ssaO, ssaS, sseB* and *sspH1* which were not present in *S.* Dublin (Figure 3.21). SPI2 genes *sopE, ssrA* and *ssrB* were identified in *S.* Dublin isolates but not *S.* Typhimurium isolates (Figure 3.21). The absence of *ssrA* and *ssrB* from *S.* Typhimurium isolates does not make biological sense because these genes are essential for the expression and function of SPI2, yet the *S.* Typhimurium isolates used in this study were presumed to be virulent (Delwick et al., 1999).

In *S.* Dublin isolates, the majority of the genes associated with flagellar structure, regulation and function were present, other than the flagellar filament genes *fliC* and *fliD*. In total, six flagellar genes were identified in *S.* Typhimurium isolates, seven times fewer genes compared to *S.* Dublin isolates. Many of the *S.* Typhimurium isolates are presumed to be virulent owing to the fact that they were isolated from humans or animals, so the finding that they may be non-motile does not make biological sense. Similarly, chemotaxis gene sequences for *cheA, cheB, cheR, cheW, cheY*, *cheZ* and *tar/cheM* were present in all isolates of *S.* Dublin, but only *cheW* and *cheY* sequences were present in *S.* Typhimurium isolates (Figure 3.21). A single outer membrane protein gene sequence (*ompA*) was present in *S.* Typhimurium isolates, whilst three (*ompD, apE* and *safA*) were present in *S.* Dublin isolates.

All isolates irrespective of serovar and origin of isolation had homologous sequences for complete fimbrial *lpf* operons, and *S.* Dublin isolates also had a complete *sti* fimbrial cluster. Elements of gene clusters associated with four fimbrial operons in total (two γ-1, one π and one κ type) were identified in *S.* Typhimurium isolates, compared to fourteen (five γ-1, two γ-3, three γ-4, three π and one κ type) in *S.* Dublin. In total, almost four times more fimbrial genes were identified in *S.* Dublin isolates compared to *S.* Typhimurium isolates. The complete *pef* fimbrial operon (*pefBACD*), encoded on a

Salmonella virulence plasmid, was significantly associated with livestock isolates in *S.* Typhimurium. However, only *pefB* was identified in *S.* Dublin isolates and this was present irrespective of origin of isolation. This contradicts previous findings which demonstrated high levels of similarity between the fimbriae present in *S.* Dublin and *S.* Typhimurium so is unlikely to be correct (Yue et al., 2012).

Homologous sequences for genes encoded by mobile genetic elements were more abundant in *S.* Dublin isolates compared to *S.* Typhimurium, largely due to the presence of *pilMNOPQRSTUVV2K* indicative of plasmid pSTM709 in five isolates (Figure 3.21). Both *S.* Dublin and *S.* Typhimurium isolates had homologous sequences for phage-associated gene *grvA*. Plasmid associated gene *mig*-5 sequences were identified in all *S.* Dublin isolates, whilst phage gene *sodC1* and plasmid gene *rck* were identified in the majority of *S.* Typhimurium isolates (Figure 3.21).

S. Dublin and *S.* Typhimurium isolates were relatively similar in the presence and absence of curli fibre genes, with sequences encoding *csgA, csgB, csgD, csgE, csgF* and *csgG* present irrespective of serovar. The *csg* operon was not intact in *S.* Dublin isolates however, with homologous sequences for *csgC* being "absent", despite the VFDB hosting sequences for *csgC* in *S.* Dublin at the time of analysis. It is unclear as to whether this is "absent" from the operon or an erroneous result. Homologous sequences for *csgC* were identified in *S.* Typhimurium, and duplicate sequences for *csgE*, *csgF* and *csgG* were also identified in one *S.* Typhimurium isolate.

S. Dublin and *S.* Typhimurium isolates did not share any genes in the outer membrane protein category, but both *shdA* and *sinH* adherence genes were identified in both serovars, with only *sinH* being present in all isolates of both serovars (Figure 3.21). *pagN* and *siiE* were only identified in *S.* Dublin isolates but were present in all isolates of this serovar (Figure 3.21). More genes associated with ferric uptake were identified in *S.* Typhimurium isolates compared to *S.* Dublin isolates

(Figure 3.21). Ferric uptake genes *iucA, iucB, iucC, iucD* and *iutA* were identified in one *S.* Typhimurium isolate, increasing the number of ferric uptake genes in this category for *S.* Typhimurium more generally. However, *entA, entB, entC, entE, entS, fepA, fepB, fepC, fepD,* and *fepG* were not identified in *S.* Dublin isolates and were present in all *S.* Typhimurium isolates (Figure 3.21). Homologous sequences for *iroB, iroC* and *iroN* were not present in a total of five *S.* Typhimurium isolates, but were present along with *iroD* and *iroE* in all but one *S.* Dublin isolates (Figure 3.21). *fur* was also identified in all but one *S.* Dublin isolate, which was not identified in *S.* Typhimurium isolates (Figure 3.21). It is unlikely that these findings regarding the presence or absence of iron uptake genes are correct, as previous work has shown *ent, fep* and *iro* genes to be essential for intestinal persistence in mice (Nagy et al., 2013).

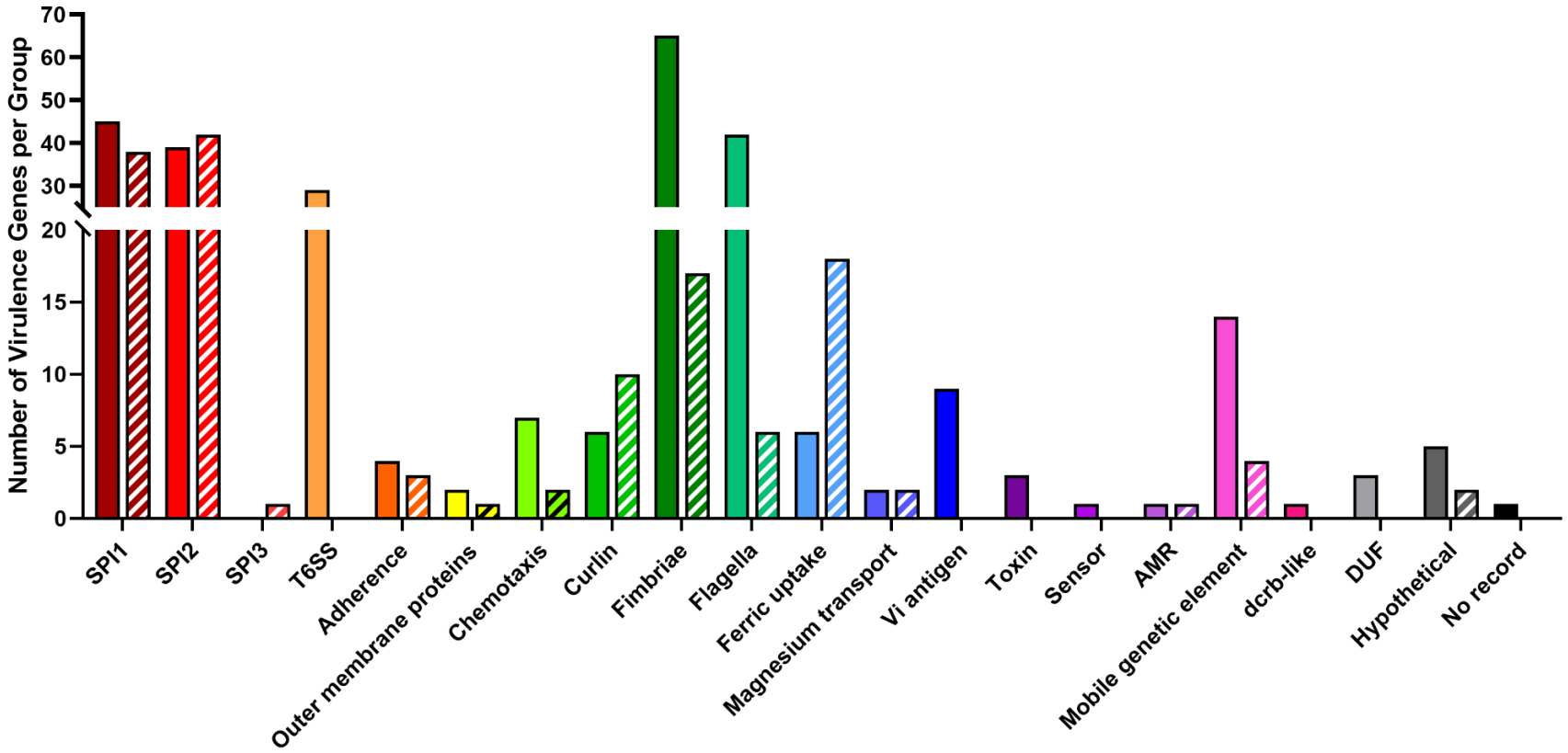


Figure 3.21 - Number of genes within the 26 functional categories identified in 266 *S.* Dublin (plain bars) and 266 *S.* Typhimurium (hatched bars) isolates. The identification of virulence factors was completed using the VFDB (Virulence Factor Database, Bo Liu et al., 2019), where genes with >90% sequence homology were classed as being "present". Investigation of the function of each virulence factor was completed using NCBI Gene and NCBI Protein and genes were grouped based on these functions. The number of genes in each functional group includes any duplicates identified. Total number of individual virulence factors (n) = 317

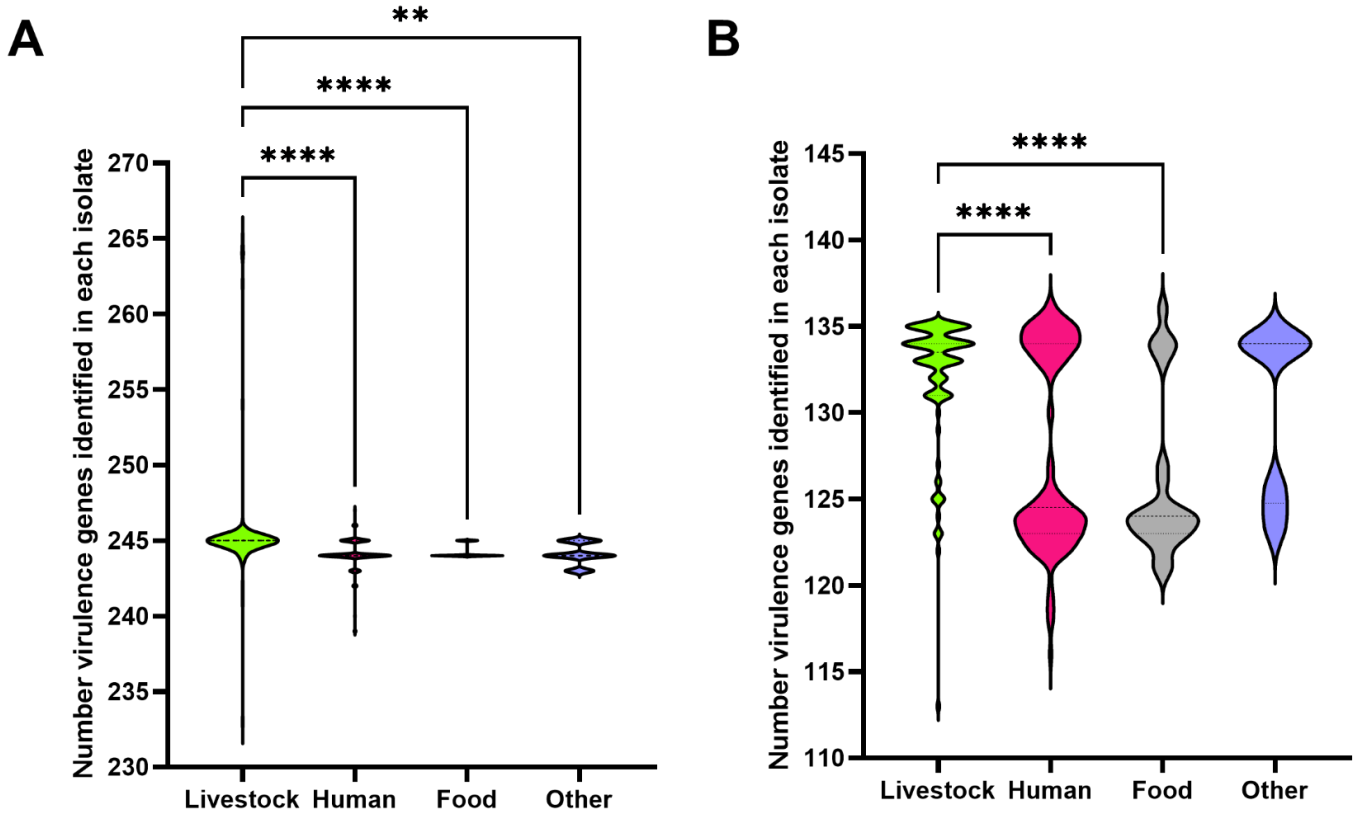


Figure 3.22 - Violin plots of number of virulence genes in each isolate of (A) 266 *S.* Dublin and (B) 266 *S.* Typhimurium isolates, split into the isolates origin of isolation. The identification of virulence factors was completed using the VFDB (Virulence Factor Database, Bo Liu et al., 2019), where genes with >90% homology were classed as being "present". Dunn's Multiple Comparison Tests were used to evaluate if there were differences between the distribution of the number of virulence genes across the different isolates grouped by their origins of isolation. ** = P<0.005, **** = P<0.00005.

3.4 - Discussion

Compared to the information available for *S.* Typhimurium, research into the virulence factors of *S.* Dublin is limited. Therefore, understanding what is representative of *S.* Dublin virulence in the UK is highly difficult without first understanding this population as a whole.

The phylogenetic alignment based on the core genome of *S.* Dublin isolates showed little to no clustering of isolates based on their origin of isolation (Figure 3.1). The wide distribution of the 16 isolates used in prior characterisation studies indicated that these isolates were representative of a wider population of *S.* Dublin isolates. When aligned based on the accessory genome, the clear clustering based on origin of isolation potentially demonstrated that there may be elements of the genome which are host specific. This is to be anticipated because the process of microbial evolution and host adaptation is thought to occur through the acquisition and gradual degradation of different genetic elements (Langridge et al., 2015). The fact that there were relatively few virulence genes significantly associated with livestock or human isolates implies that genes other than those associated with virulence are likely to be the source of this clustering and should be explored further.

Much of the bioinformatic work using the VFDB was hindered by the fact that many of the results obtained using the VFDB did not make biological sense. For example, the fact that *S.* Dublin isolates from human sources were significantly less likely to harbour an *ssaU* sequence of more than 90% similarity is extremely unlikely to be correct. SsaU forms the SsaRSTUV complex spanning the bacterial inner membrane in the T3SS and is specifically implicated in the secretion switch from early to late-stage effector proteins (Feria et al., 2015; Riordan and Schneewind, 2008; Sorg et al., 2007; Yu et al., 2018). Deletion of *ssaU* was shown to inhibit secretion of effector proteins essential for bacterial virulence and intracellular survival (Yu et al., 2018). Studies in which the *ssaB-U* operon is mutated or

deleted in *S.* Typhimurium demonstrate bacterial virulence attenuation, a foreseeable outcome as this comprises the majority of the needle complex (Cox et al., 2016; Sabag-Daigle et al., 2016). Mutations to *ssaU* specifically have demonstrated the importance of this gene in the virulence of *S.* Typhimurium and *S.* Gallinarum in free-living amoeba and chickens respectively (Bleasdale et al., 2009; Jones et al., 2001). A functional SPI2 is essential for virulence and persistence and is activated once the bacteria has entered a host cell and resides in the SCV (Shea et al., 1996; Valdivia and Falkow, 1997). This wealth of evidence suggesting that SsaU is absolutely essential for the functionality of SPI2 and bacterial virulence suggests that this finding is an artefact of the use of the VFDB. Another study utilising the VFDB identified *ssaU* as a "variable" gene with a prevalence of less than 85% amongst more than 1,077 *Salmonella* isolates of different serovars from Brazil (dos Santos et al., 2021). The authors did not consider this to be a potentially erroneous result, instead suggesting that this may be indicative of the process of host adaptation, as many of the isolates were still implicated in clinical illness in humans and animals (dos Santos et al., 2021). However, due to the importance of SsaU in the function of the SPI2 T3SS, it is probable that its "absence" is an incorrect finding. It is more likely that the *ssaU* sequences found in *S.* Dublin differ sufficiently to those in *S.* Typhimurium (used in the VFDB) that the identity scores fail to meet the threshold to be considered "present", and that the VFDB is limited by the lack of information available for *S.* Dublin. Further work investigating the differences between *ssaU* sequences in different serovars should be conducted, including deciphering any alterations in amino acid sequence and any downstream impact on protein folding which could impact the T3SS needle complex. It is possible to characterise the functionality of the SPI-2 secretion system without *ssaU* or with mutated *ssaU* sequences by characterising the secretion of SPI-2 effectors. Additionally, it would be useful to investigate the impact of these differences in *ssaU* sequence on the virulence and persistence of the bacteria *in vitro* in species-specific models, including intestinal cells, macrophages and reproductive cells in the

context of bovine abortion. As isolate L 2160/17 appears to harbour a sequence heterologous to that of the other isolates in the previous studies, this could be used as a comparative isolate without the need to construct mutants, although a PCR confirming the identity of this gene would be beneficial.

The VFDB identified some unusual groups of genes in five *S.* Dublin isolates from livestock sources which clustered closely together in the accessory alignment. The VFDB-inferred identification of twelve pilus genes and nine vi antigen genes may indicate the presence of SPI-7, a pathogenicity island known to harbour the *viaB* locus encoding *pil*, *vex* and *tvi* (Pickard et al., 2003). SPI-7 has been identified in *S.* Dublin before and is more often observed in isolates associated with livestock (Mohammed et al., 2017; Manal Mohammed, Vignaud, and Cadel-Six, 2019; Pickard et al., 2003; Seth-Smith et al., 2012). *tviA* sequences were not discovered in these isolates, though at the time of analysis, the VFDB only contained *tviA* sequences for serovars Typhi and Paratyphi so these sequences may not have been similar enough to those present in the *S.* Dublin isolates for this gene to be considered "present". The acquisition of this pathogenicity island is most likely a result of horizontal gene transfer, supported by the fact that four of the isolates were collected in 2007-2008 and the other ten years later in 2017 indicating its infrequency in the UK population (Pickard et al., 2003).

Homologous sequences for the *spvC* gene encoding an anti-inflammatory effector protein were identified in all five of these isolates but no other *S.* Dublin isolates (Guiney and Fierer, 2011). Additionally, *spvD* was not identified in any of the *S.* Dublin isolates, and *spvA, spvD* and *spoS* were not identified in any of the *S.* Typhimurium isolates. *spvC* is located in the IncF-type *Salmonella* virulence plasmid within the *spvABCDR* locus, along with regulator *rpoS* and is essential for *S.* Dublin virulence in calves (Libby et al., 1997). This region is highly conserved among *Salmonella* and *spvC* in particular is involved in inhibiting intestinal inflammatory responses

which promotes systemic infection (Zuo et al., 2020). Therefore, it is unlikely that the VFDB results are correct for either *S.* Dublin or *S.* Typhimurium. Diverging lineages in *Salmonella* virulence plasmids exist, with *S.* Typhimurium harbouring pSENV which is phylogenetically distinct from the *S.* Dublin pSDUV, and variation in plasmids has also been observed within the same serovar (Chu et al., 2008; Feng et al., 2012). However, at the time of analysis, the VFDB contained records for all of the *spv* locus genes in both *S.* Dublin and *S.* Typhimurium, so it was surprising that these loci were not identified and demonstrates another limitation of using the VFDB (Liu et al., 2019).

Homologous sequences for *rck*, encoding the resistance to complement killing protein which prevents complement-induced bacterial lysis were identified in *S.* Typhimurium isolates and not *S.* Dublin isolates, in keeping with the findings of others (Feng et al., 2012; Koczerka et al., 2021). Similarly, the *S.* Typhimurium virulence plasmid pSENV also harbours the *pefBACD* fimbrial operon, another operon identified in the *S.* Typhimurium isolates which are significantly associated with livestock-associated isolates (Feng et al., 2012). However, *pefB* was identified in all but one of the *S.* Dublin isolates which was inconsistent with previous findings for the pSDUV plasmid and the diverging lineages of the pSDVr and pSDVu plasmids (Chu et al., 2008; Feng et al., 2012). It is possible that the isolates used to investigate plasmids in *S.* Dublin differ substantially to those used in the present study due to geographical implications, as they were isolated in Taiwan and Canada (Chu et al., 2008; Feng et al., 2012). However, it is also possible that the findings presented here were incorrect, as has been hypothesised for a number of other results generated using the VFDB (Liu et al., 2019).

The VFDB-inferred finding that iron uptake genes were vastly different in *S.* Typhimurium compared to *S.* Dublin is also likely to be erroneous. Whilst the process and principles of pseudogenisation may indicate that serovars with broad host ranges may harbour more

genes with which to infect different host species (for example, *S.* Enteritidis harbours iron uptake genes which are functionally redundant in chickens), iron uptake is essential for survival and virulence (Wellawa et al., 2022). Therefore, the "absence" of essential iron uptake genes *entA*, *entB, entC, entE, entS, fepA, fepB, fepC, fepD,* and *fepG* in the *S.* Dublin isolates would indicate an issue in the VFDB rather than there being this stark difference between *S.* Dublin and *S.* Typhimurium due to pseudogenisation and host specificity. Similarly, a large number of fimbrial operons were identified in *S.* Dublin and not *S.* Typhimurium isolates, which contradicts previous studies demonstrating both serovars harbouring *stc, sth, stb, fim, bcf, saf, sti, stf, lpf, fae,* and *stj* operons (Yue et al., 2012). The *stj* and *fae* operons were not identified in this study but this is likely to be because there are few entries into the VFDB for *stj*, and none for *Salmonellae* for *fae* (Liu et al., 2019). Finally, there were no T6SS genes identified in the *S.* Typhimurium isolates. Whilst it was more likely that more T6SS genes would be identified in *S.* Dublin as this serovar harbours both SPI-6 and SPI-19, SPI-6 was initially identified in *S.* Typhimurium so it is unlikely that these genes were "absent" in the isolates in this study (Folkesson, Löfdahl, and Normark, 2002; Mulder, Cooper, and Coombes, 2012; Schroll et al., 2019).

3.4.1 - Limitations of the Virulence Factor Database Approach

A number of limitations of using the VFDB have been identified in this work, demonstrated by the fact that some of the results presented here do not make biological sense.

Whilst the VFDB hosts virulence gene sequences from a range of different *Salmonella* isolates of different serovars and species, there were many instances in the present study where gene sequences in *S.* Dublin isolates were compared to gene sequences identified in *S.* Typhimurium isolates. This means that some genes may have appeared to be "absent" in *S.* Dublin isolates (for example, *ssaU*) which were extremely unlikely to have been because of their

importance in bacterial virulence and survival. On the occasions when sequences were available for both *S.* Dublin and *S.* Typhimurium (for example, the *spv* locus), various genes deemed essential were still recorded as "absent". This demonstrates the need for a greater number of gene records from a larger range of serovars to be added, including duplicate entries of the same serovar to ensure different populations are represented. Additionally, many of the *S.* Typhimurium gene records were based on isolate LT2, a well-characterised strain isolated in the 1940s (Lilleengen, 1948). Genetic shift occurs over time in laboratory isolates which can mean they are no longer representative of the currently circulating population, including within the same serovar (Liu et al., 2003). The basis for the VFDB algorithm is unclear so it is difficult to understand how or why erroneous results may be generated outside of the assumptions set out here.

A group investigated the presence of different virulence factors in non-typhoidal *Salmonella* isolates associated with clinical disease in humans in The Gambia and noted in a pre-print that a number of essential genes involved in iron uptake were seemingly absent (Darboe et al., 2020). In the published paper, this finding was omitted which may indicate that the researchers thought this could be an error (Darboe et al., 2022). Interestingly, many of those iron-related genes were also "absent" in the work presented here, including *entA, entB, entE, fepC* and *fepG*.

Many researchers are using the VFDB as a powerful tool for investigating the different virulence factors present in *Salmonella* and other bacteria. Despite its wide usage, there does not appear to be a justified consensus on appropriate identity thresholds to use when investigating virulence factors using the VFDB. Various groups investigating *Salmonella* have used identity thresholds of between 95% and as low as 70% (González-Torres et al., 2023; Seribelli et al., 2021; Wang et al., 2022). Using lower thresholds may be beneficial for sequences with poorer coverage which, whilst not ideal,

may be the best option for under-researched and therefore under-represented serovars. However, it is important to consider this as a potential factor for inaccuracies in presence/absence studies. All results should be validated using alternative bioinformatic or laboratory-based methodology. A large study using well-annotated *Salmonella* genomes may be useful in understanding the validity of the VFDB findings, as this would demonstrate where there is information missing from the database and aid in understanding where results can be taken as being more reliable.

3.5 - Summary

Several key genetic elements of interest were found to be shared by both *S.* Dublin isolates and *S.* Typhimurium isolates of livestock origin, indicating that there may be some host-specific virulence adaptations which can be further investigated. The 16 *S.* Dublin isolates used in the previous characterisation chapter were found to be similar to a wider population of isolates from the UK. Therefore, they can be used in further virulence studies with the knowledge that they should be representative of circulating and therefore clinically relevant strains.

Whilst these computational analyses aid in predicting the virulence of different isolates, it is only possible to infer from this data how bacteria may behave *in vivo*. This can be determined not only by the genetic virulence of the bacteria but by a range of different host interactions too. It is therefore important to characterise the survival of the bacteria in different host environments as models of an infection scenario.

Chapter 4 - Determining *S.* Dublin survival in BCECs and bovine whole blood as models of host systems

4.1 - Introduction

An essential part of the progression from initial enteric infection to an abortion event is the dissemination of *S.* Dublin from the gastrointestinal tract to the reproductive tract and the subsequent infection of the placentomes. *S.* Dublin has been isolated from the placenta of experimentally infected cattle, some of which aborted, as well as liver, spleen, lung and lymph node indicating systemic dissemination (Hall and Jones, 1977). However, it is not clear how *S.* Dublin penetrates through the different layers of the placentome in order to initiate an abortion and there is no specific experimental evidence to demonstrate systemic dissemination of *S.* Dublin in cattle within monocytes or macrophages. Additionally, the presence of lymphatic vessels in the placenta is questionable, with the available literature stating that vessels have not been observed in human placentas (Becker et al., 2020; Castro, Tony Parks, and Galambos, 2011). There are no reports available detailing the presence of lymphatic vessels in the placentome, meaning that the theory for lymphatic dissemination may be questionable when linked to understanding *S.* Dublin as an abortifacient pathogen (Pullinger et al., 2007). Therefore, alternative mechanisms for bacterial dissemination towards the reproductive tract need to be hypothesised and explored. To understand these elements of *S.* Dublin pathogenesis, it would be beneficial to investigate the virulence of *S.* Dublin in reproductive tissues and understand the survival of *S.* Dublin in whole bovine blood.

4.1.1 - Bovine Caruncular Epithelial Cells

BCECs were isolated from the placentome of a deceased pregnant cow, where the fetal and maternal tissues were separated to leave caruncular cells (Figure 4.1) (Bridger, Menge, et al., 2007). The BCEC cell line maintains the epithelial phenotype (characterised by the presence of epithelial cytokeratin and tight-junctional protein zona occludens-1), and vimentin which is absent in a non-pregnant bovine uterus (Bridger, Haupt, et al., 2007). The ability of BCECs to express heparanase, a protein thought to be important in the context of retained fetal membranes in cattle, has been described and demonstrates again the ability of BCECs to retain the placentome phenotypes (Hambruch et al., 2017). BCECs express CK18 in co-culture with bovine fibroblasts, an important marker of the process of cellular polarisation necessary for implantation during pregnancy (Haeger et al., 2015). BCECs have been used previously to investigate different pathogens of the bovine reproductive tract, including *Listeria monocytogenes* (Blanchard et al., 2019, 2020; Rupp et al., 2015, 2017), *Neospora caninum* (Jiménez-Pelayo et al., 2019b, 2019a) and Schmallenberg virus (Somerwill, 2014). Work with Schmallenberg virus also included producing co-cultures of BCECs with trophoblast cells, demonstrating the ability of BCECs to form three-dimensional structures in culture (Somerwill, 2014). Placental drug susceptibility has also been investigated using BCECs (Waterkotte et al., 2011). Due to the BCECs ability to maintain a placental phenotype and their prior use investigating infectious disease of the bovine reproductive tract, these cells were chosen for the investigation of *S*. Dublin virulence in the reproductive tract.

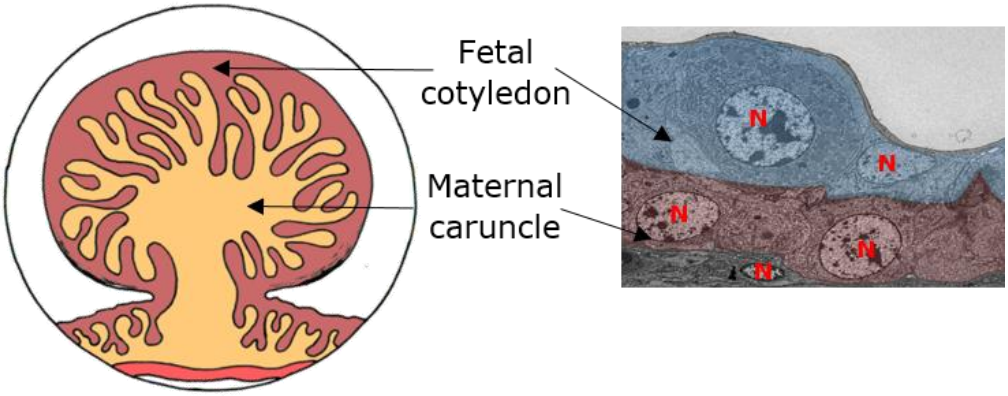


Figure 4.1 – Schematic and TEM image of the caruncular-cotyledonary interface in the bovine placentome. Transmission Electron Microscopy image provided by Karl Klisch, University of Zurich, overlayed with colour to emphasise cell layers (blue indicating fetal cells, pink indicating maternal cells). N indicates cell nucleus.

4.1.2 - Whole blood survival assays

Salmonellae have been shown in many species to be able to infect and persist within monocytes and macrophages and it has thus been assumed that this is the predominant route of dissemination. *S.* Dublin can infect bovine macrophages and these immune cells increase in number in the placentomes during pregnancy, providing the opportunity for dissemination (Miyoshi and Sawamukai, 2004; Watson et al., 2000). However, *S.* Dublin has not been isolated from bovine macrophages in cattle with bacteraemia so alternative methods of dissemination could be considered. *S.* Dublin has been isolated from the jugular blood of experimentally infected calves (Pullinger et al., 2007). Colonisation of other organs appears to precede colonisation of the placentome, so an alternative mechanism for bacterial survival could involve free-living bacteria disseminating from other tissues towards the placentome. Furthermore, vascularisation in the placentome is highly convoluted, providing an opportune site for bacterial adhesion and invasion of the vascular endothelium (Betbeze and McLaughlin, 2002).

Whole blood survival assays have been used to investigate the virulence of different bacteria and bactericidal/fungicidal capabilities of blood in a variety of infectious species and host species, including *Candida* spp., *Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis, Neisseria meningitidis, Listeria monocytogenes* and *Streptococcus* species (Echenique-Rivera et al., 2011; Graham et al., 2005; Mereghetti et al., 2008; Sreekantapuram et al., 2020; Tena et al., 2003; Toledo-Arana et al., 2009). Many of these studies also include characterisation of the host response via quantification of cytokine production as well as transcriptomic analysis of the various immune cell types present. Whilst limited, whole blood studies have been used in *Salmonella* serovars, specifically investigating differences between *S.* Gallinarum and *S.* Enteritidis in the blood of high and low performing chickens and the impact of *S.* Enteritidis vaccine strains on CD25+ gamma/delta (γδ) T cells (Braukmann, Methner, and Berndt, 2015; Sreekantapuram et al., 2021). Additionally, the presence of the Vi antigen and its impact on growth in human blood has been evaluated in strains of *S.* Typhi (Liaquat et al., 2015). Presently, work investigating *S.* Dublin survival in bovine blood has not been conducted.

4.1.3 - Aims and hypotheses

This chapter aims to determine the ability of 16 *S.* Dublin isolates (15 abortion-related, and one well-characterised laboratory strain) to infect bovine caruncular epithelial cells as a model of their virulence in the placentome. This will help to reduce the number of isolates chosen for future work investigating the host response whilst avoiding introducing further variability when the isolates may differ in their virulence. Additionally, the bacteria's ability to survive and replicate in bovine whole blood will provide an insight into how the bacteria may be disseminated.

We hypothesise that the different isolates will infect and survive within BCECs to the same extent, despite the phenotypic and genotypic differences previously characterised. We also hypothesise

that the four isolates chosen for whole blood survival studies will survive to similar extents in the blood.

4.2 - Materials and Methods

4.2.1 - BCEC Culturing

Bovine caruncular epithelial cells were kindly provided by Christiane Pfarrer of the University of Hannover, Germany (Bridger, Haupt, et al., 2007).

The cells were preserved in freeze medium (Sigma Aldrich, United Kingdom) in liquid nitrogen. At least two weeks prior to experiments, cells were thawed and cultured in BCEC Medium (Table 4.1) at 37°C and 5% CO_2 in T25 (25cm^2) Nunc EasYFlask Cell Culture Flasks (Thermofisher, United Kingdom). Routine passage and expansion of cultures occurred when the cells reached 80-90% confluence, at which point the BCEC medium was removed, and the cells were washed with pre-warmed PBS without Mg^{2+}/Ca^{2+} (Sigma Aldrich, United Kingdom). The PBS was removed and replaced with PBS containing 0.5% Trypsin-EDTA (Sigma Aldrich, United Kingdom). The cells were incubated at 37°C and 5% CO_2 for between 10 and 15 minutes. Once the cells had detached from the culture flask, fresh pre-warmed BCEC Medium was added and mixed by gentle pipetting before being transferred into new T75 (75cm^2) flask. Further expansion proceeded once the cells reached 80-90% confluence as previously described and were diluted at a ratio of either 1:2 or 1:3.

24 hours prior to infection, the cells were passaged into 12 well plates at approximately 7.0 x 10^5 cells/well and incubated overnight to colonise the wells at 37°C and 5% CO_2. Two hours prior to infection, the cell culture medium was aspirated, and the cells were washed with pre-warmed PBS. The PBS was removed and replaced with BCEC medium without antibiotics, and the cells were incubated for at least two hours prior to infection at 37°C at 5% CO_2 prior to infection.

Table 4.1 – BCEC culture medium supplements.

Supplement	Manufacturer	Final concentration
Fetal Calf Serum	Sigma Aldrich (UK)	10% (v/v)
L-glutamine	Sigma Aldrich (UK)	10mg/ml
Penicillin	Sigma Aldrich (UK)	100U/ml
Streptomycin	Sigma Aldrich (UK)	0.1ug/ml

Dulbecco's Modified Eagles Medium/Hams F-12 (Corning, United States) medium supplemented with the detailed components to produce a complete medium suitable for the culture of BCECs (Bovine Caruncular Epithelial Cells).

4.2.2 - Bacterial culturing and producing inoculum

One day before infection, two or three colonies of each isolate were picked and used to inoculate 5ml of sterile NB. These cultures were grown overnight in an orbital shaking incubator at 37°C. The following day, 1ml of each culture was transferred into 4ml of fresh NB, and incubated for two hours at 37°C in an orbital shaking incubator, and diluted to the desired MOI in BCEC medium without antibiotics. The CFU/ml was determined for each well by sampling the lysate, diluting and plating using the method described by (Miles, Misra, and Irwin, 1938). 10µl of each dilution was plated in triplicate and allowed to run vertically down the plate to increase the surface area available to count (Figure 4.2). Agar plates were incubated overnight at 37°C until colonies could be counted. Bacterial colonies were counted and calculated as CFU/ml from these diluted counts. Data were compiled in Microsoft Excel 2016 and analysed using GraphPad Prism 8.1.

4.2.3 - Gentamycin sensitivity testing

Prior to use in infection studies, the 16 *S.* Dublin isolates were tested for their sensitivity to gentamycin at 100µg/ml, similar to gentamycin protection assays (Elsinghorst, 1994). Antibiotic-free BCEC Medium was prepared and included Dulbecco's Modified Eagle's Medium/Hams F-12 (Corning, United States), 10% Fetal Calf Serum (Sigma Aldrich, United Kingdom) and 10mg/ml L-glutamine (Sigma Aldrich, United Kingdom). Overnight bacterial cultures were prepared as described in 4.2.2 - Bacterial culturing and producing inoculum, diluted 1:10 in sterile NB and incubated for two hours at 37°C in an orbital shaking incubator. 450µl of BCEC medium with 100µg/ml gentamycin was

added to individual wells of a 24-well cell culture plate (Nunclon Delta-Surface, Thermofisher United Kingdom). 50µl of the bacterial cultures were added to these wells, containing approximately 2 x 10^6 CFU/ml. These cultures were left to incubate at 37°C in 5% CO_2 for two hours, before samples were taken for serial dilution in sterile PBS and plating on NA as described previously (Figure 4.2) (Miles, Misra, and Irwin, 1938). Agar plates were incubated at 37°C for 19 hours.

4.2.4 - Infection of BCECs

Antibiotic-free medium was aspirated from the BCECs, and the inoculum diluted in antibiotic-free BCEC medium was added at an MOI of 1, 10 or 50. The infected cells were incubated at 37°C and 5% CO_2. After 1 hour, the medium was removed, and replaced with medium containing 100µg/ml gentamycin (Fisher Scientific UK Ltd) in order to kill any extracellular bacteria.

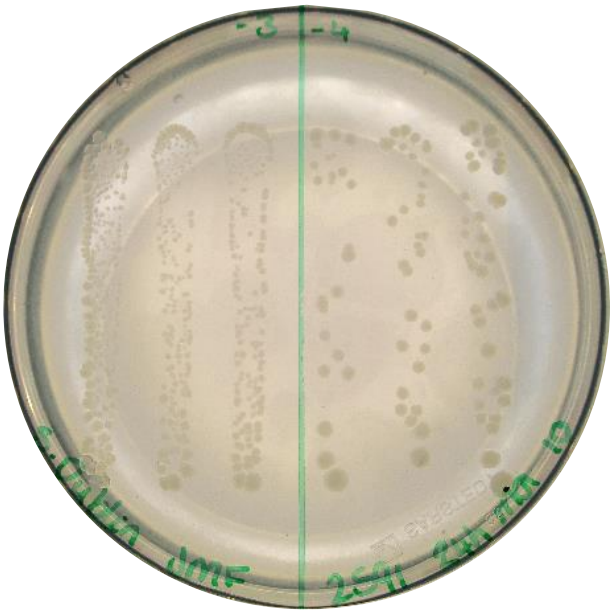


Figure 4.2 – Example of *S.* Dublin colonies on NA after serial dilutions using the Miles and Misra method (Miles, Misra, and Irwin, 1938)**.** *S.* Dublin isolates were grown overnight in NB (nutrient broth) and serially diluted in sterile PBS (phosphate buffered saline). 10µl droplets were dropped in triplicate onto NA (nutrient agar) and allowed to run down the plate to provide a larger surface area to count the colonies.

4.2.4.1 - Infection

Following 1 hour of incubation with 100ug/ml gentamycin, the medium was removed, and cells were washed with pre-warmed, sterile PBS. Cells were then lysed using 100µl 0.5% triton for 20 minutes on ice.

4.2.4.2 - 24h, 48h and 72h Infections

Following 1 hour of incubation with 100µg/ml gentamycin, the medium was removed and replaced with BCEC medium containing 5µg/ml gentamycin. The cultures were then left for a further 22h, 46h or 70h before being lysed using the same method as described in 4.2.3.1.

The percentage of adherent BCECs was estimated by visual inspection at each time point to assess the suitability of the MOI, and to assess cell viability over the longer time courses.

4.2.5 - Whole blood survival

Bovine blood was provided for this study under project license PPL 30/3383, collected from healthy cattle not in receipt of antibiotics and without prior history of salmonellosis. The blood was collected from the jugular vein into lithium heparin vacutainers (Fisher Scientific) and used within 30 minutes of collection. Blood from the same animal was pooled to ensure the same concentration of anticoagulant through the sample. 10ml aliquots were placed into 15ml falcon tubes, placed on a blood roller, and incubated at 37°C and 5% CO_2 prior to infection.

Four *S.* Dublin isolates were chosen based on characterisation previously carried out (Table 4.2). As described previously, bacteria were cultured overnight in 5ml NB in an orbital shaking incubator at 200 RPM and 37°C (4.2.2 - Bacterial culturing and producing inoculum). The following day, 1ml of this overnight culture was diluted into 4ml sterile NB and incubated for 2h in an orbital shaking incubator at 200 RPM and 37°C. A sample of this 2h culture was

diluted into 10ml of fresh bovine blood to an estimated $1x10^6$ CFU/ml, an inoculum widely used in the literature.

Five minutes post-inoculation, 30ul of blood was sampled, serially diluted, and plated onto NA. This sampling, dilution and plating was repeated at 30-minute intervals post-infection for the next five hours and at 24 hours post-inoculation. The plates were incubated at 37°C overnight until colonies could be counted (Figure 4.2).

Table 4.2 – *S.* Dublin isolates chosen for use in whole blood survival studies.

Isolate	Origin of isolation	Growth Phase	Virulence Profile	VFDB-inferred presence/ absence of virulence genes		
				ssaU	*fimW*	*stfE*
L 2160/17	Abortion	Monophasic	VP19	-	+	-
L 2185/17	Abortion	Biphasic	VP1	+	+	-
L 2591/17	Abortion	Monophasic	VP1	+	+	-
2229	Diarrhoea	Monophasic	VP4	+	-	+

All isolates were of bovine origin. +/- indicates whether virulence genes *ssaU, fimW* and *stfE* were "present" as inferred by the previous bioinformatic analysis presented in Chapter 2 using the VFDB (Virulence Factor Database, Bo Liu et al., 2019).

4.2.6 - Statistical analysis

Statistical analysis was performed using GraphPad Prism (v. 8.1). Kruskal-Wallis tests were used to identify variation within data sets. Dunn's Multiple Comparisons tests identified specific differences between isolates, time points and MOI. Both test methods were non-parametric because normal distribution could not be assumed. The rate of bacterial cell death was calculated by fitting a logistic growth curve to the CFU/ml obtained during the experiment multiplied by -1, where the output k represents the maximum rate in the curve (Equation 2.1). The rate of bacterial cell death was also analysed using Kruskal-Wallis and Dunn's Multiple Comparisons tests to identify any differences between the isolates.

4.3 - Results

The ability of *S.* Dublin isolates to enter and replicate within BCECs was investigated as a measure of bacterial virulence. A range of MOIs

and time points were used initially in pilot studies to discern the most appropriate timelines and MOIs for future work.

4.3.1 - *S.* Dublin isolates are sensitive to gentamycin

Future infection studies would rely on the *S.* Dublin isolates being sensitive to gentamycin, as these experiments would infer whether the bacteria were intracellular and therefore protected from the antibiotic. Therefore, it was important to determine whether the isolates were sensitive to gentamycin before their use in infection studies. All *S.* Dublin isolates were sensitive to gentamycin at 100mg/ml, demonstrated by there being no visible cultures on NA plates after 19 hours in culture (data not displayed).

4.3.2 - Pilot study - Infection of Bovine Caruncular Epithelial Cells with varied MOIs

Isolates L 1938/17, L 2591/17 and reference strain 2229 were used to carry out infection studies with MOIs of 1, 10 and 50 to determine a suitable infectious dose for future experiments at 2h and 24h post infection. Plating of the inoculum confirmed that the actual MOI was representative of the desired MOI of 1 and 10, but an MOI of 50 was closer to an MOI of 100 (Figure 4.3 A and Table 4.3).

At 2h, the number of bacteria recovered at all MOIs showed an approximate ten-fold difference (MOI 1 average being $2.2x10^3$, MOI 10 being $3.5x10^4$ and MOI 50 being $1.3x10^5$) between each other (Figure 4.3 B). There was a hundred-fold decrease in bacterial numbers compared to the inoculum which was consistent across all isolates and MOIs. At 24, bacterial recovery was similar for all MOIs, with MOI 1 average being $103x10^7$, MOI 10 being $2.4x10^7$ and MOI 50 being $1.2x10^7$ (Figure 4.3 C). This indicated approximately a 10^4, 10^3 and 10^2 increase in MOI 1, MOI 10 and MOI 50 respectively from 2h (Figure 4.4). Data for an MOI of 1 at 24h in isolate 2229 is absent due to colonies not being countable.

Over the course of 24h, an MOI of more than 10 is detrimental to the cells, as up to 50% of cells detached during the experiment, compared

to that of the lower MOI where wells were still 100% confluent (Table 4.4). Based on the findings in this pilot study, an MOI of 1 and 10 were used in subsequent studies.

Table 4.3 – Actual MOI of *S.* Dublin inocula used in infection study using BCECs. Different MOIs were investigated to discern the most appropriate for use in BCEC infection experiments, however it was not always possible to accurately produce an inoculum with the desired MOI.

Isolate number	Desired MOI		
	MOI 1	MOI 10	MOI 50
L 1938/17	1.2	14.6	120.8
L 2591/17	1.4	12.6	88.3
2229	1.8	15.5	73.3

MOI = Multiplicity of Infection

BCEC = Bovine Caruncular Epithelial Cells

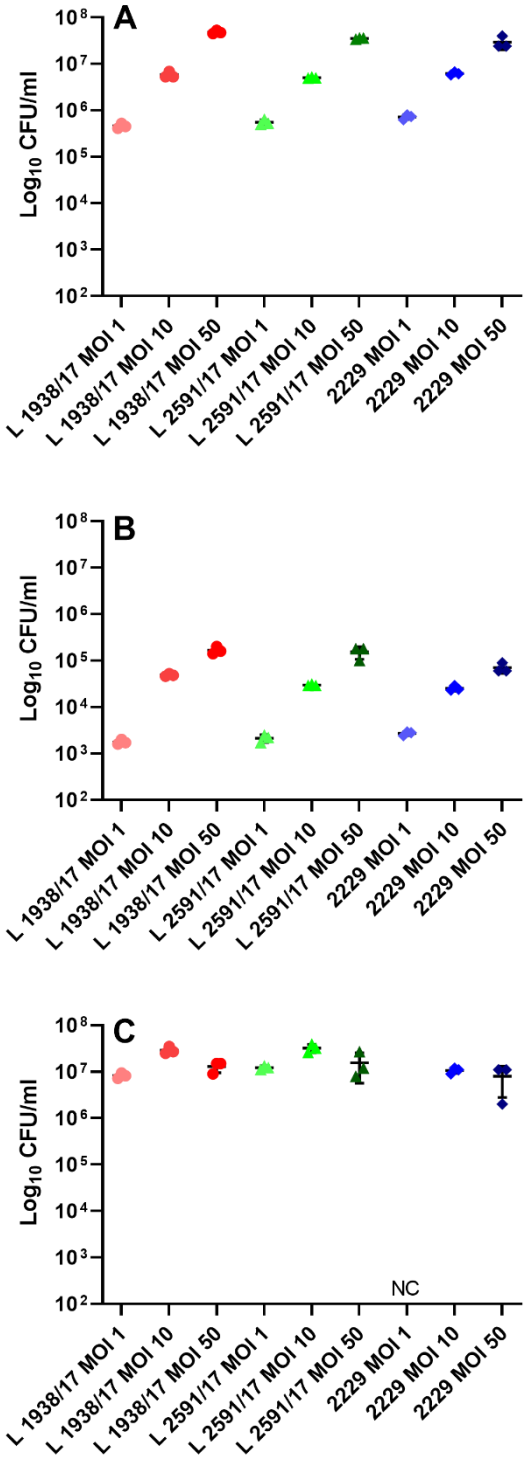


Figure 4.3 – *S.* Dublin inoculum (A) used in BCEC pilot infection studies and recovery of bacteria 2h (B) and 24h (C) post-infection. Bovine caruncular epithelial cells (BCECs) were infected with isolates L 1938/17, L 2591/17 and 2229 at three different multiplicities of infection (MOIs), 1, 10 and 50. BCECs were lysed after 2 and 24h and the presumptive intracellular bacteria were sampled, serially diluted, and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938). Samples were plated in triplicate and presented as individual points. Data for isolate 2229 at an MOI of 1 after 24h is absent as colonies were not countable. n=1

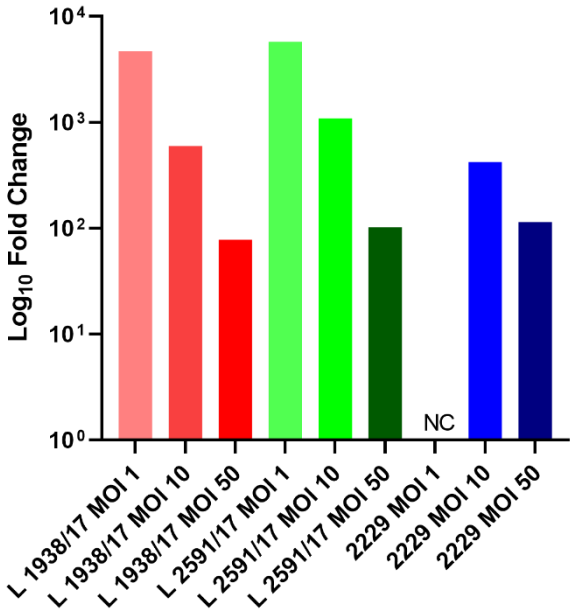


Figure 4.4 - Fold-change in *S.* Dublin recovered from infection of BCECs between 2 and 24h post-infection. Bovine caruncular epithelial cells (BCECs) were infected with isolates L 1938/17, L 2591/17 and 2229 at three different multiplicities of infection (MOIs), 1, 10 and 50. BCECs were lysed after 2 and 24h and the presumptive intracellular bacteria were sampled, serially diluted, and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938). The fold change between the bacteria recovered after 2h of infection and bacteria recovered after 24h of infection was calculated and presented. Data for isolate 2229 at an MOI of 1 after 24h is absent as colonies were not countable. n=1

Table 4.4 – Estimated percentage of BCEC confluence after 24h infection with *S.* Dublin at MOIs of 1, 10 and 50.

Isolate number	MOI 1	MOI 10	MOI 50
L 1938/17	100%	60%	50%
L 2591/17	100%	70%	60%
2229	100%	80%	60%

Bovine caruncular epithelial cells (BCECs) were infected for 24h and percentage cell attachment was assessed as an indicator of cell survival compared to "mock-infected" BCECs. "Mock infected" BCECs underwent identical treatment as the infected cells, but bacteria were not added to the cells. MOI = multiplicity of infection.

4.3.3 - Pilot study - Infection of Bovine Caruncular Epithelial Cells over the course of 72 hours

In order to discern an appropriate timeline for infection studies, a pilot study was conducted over the course of 72 hours with an MOI of 1. Isolates L 1941/17, L 2100/17, L 2160/17, L 2162/17, L 2185/17, L 2517/17, L 2591/17 and 2229 were chosen based on their relatedness

in the MLST clades, and differences in their SPI-2 gene similarity, inferred from results generated using the VFDB (Liu et al., 2019). In general, the inocula were consistent for all isolates except L 2185/17 and L1941/17 which varied significantly compared to isolate 2229 in this experiment (P=0.05) (Figure 4.5).

A clear characteristic pattern is seen across all isolates over the 72h time course. Following initial infection, the number of bacteria recovered from the cells after 24h of infection reached on average $1.15X10^7$ CFU/ml, and decreases in approximately ten-fold increments over the remainder of the time course, with an average of $4.59x10^5$ CFU/ml at 72h (Figure 4.7 A-H, Figure 4.8).

Cell density was visually assessed during this study, and a mock treated control population was compared with the infected cells. With an MOI of 1, no differences were observed between the control and infected wells at 2h and 24h (Figure 4.6 and Figure 4.7). At 48h however, the cell coverage in wells infected with isolates L 1941/17, L 2100/17, L 2160/17 and 2229 decrease markedly to between 50 and 70% compared to the control population, which reached over-confluence at 210% coverage. At 72h, the cells in all infected populations reach over-confluence (120%), whilst the control well continued to grow to 150% coverage. Large numbers of detached cells were observed in all populations when over-confluence was reached (data not shown).

Based on these pilot data, an MOI of 1 and 10 will be used over the course of 24h to investigate the virulence of all of the isolates.

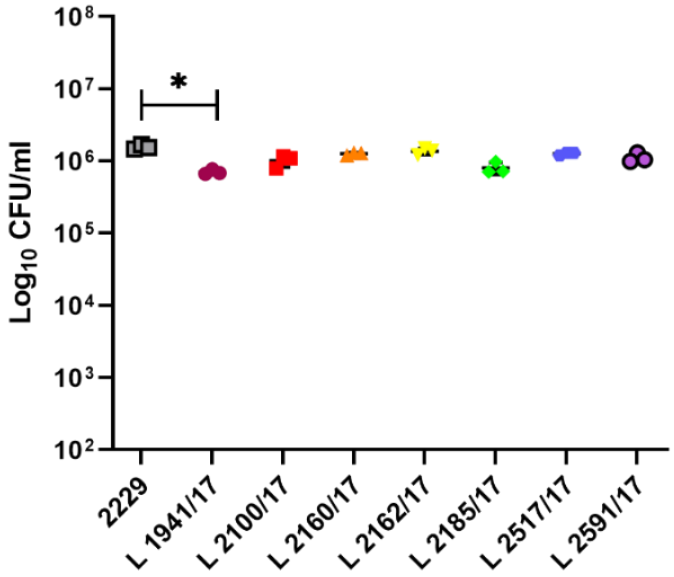


Figure 4.5 – *S.* Dublin inocula used in pilot infection study of BCECs. Bacteria were grown into log phase and diluted to a concentration of approximately $4.0x10^5$ CFU/ml, equalling a multiplicity of infection (MOI) of 1. The inocula were used to infect bovine caruncular epithelial cells (BCECs) over the course of 24h. Inocula for isolate 2229 and L 1941/17 differed significantly when statistically challenged using Kruskal-Wallis and Dunns Multiple Comparisons tests (P=<0.05). n=1.

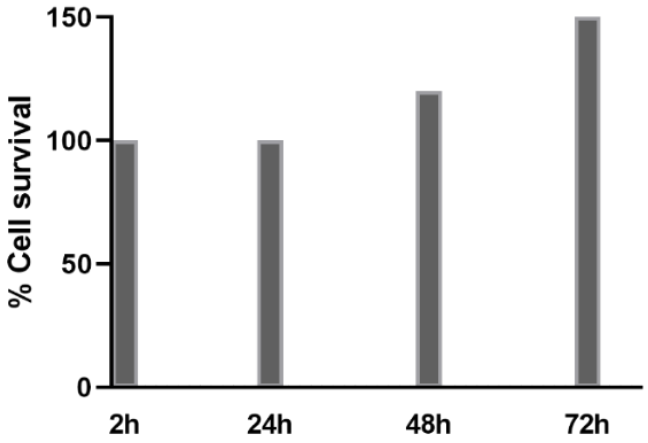


Figure 4.6 – Estimated BCEC control cell confluence over 72h. Control bovine caruncular epithelial cells (BCECs) were treated identically to infected cells, but bacteria were not added. Confluent cells are given a % cell survival with 100%, and over-confluent cells are denoted as more than 100%. n=1

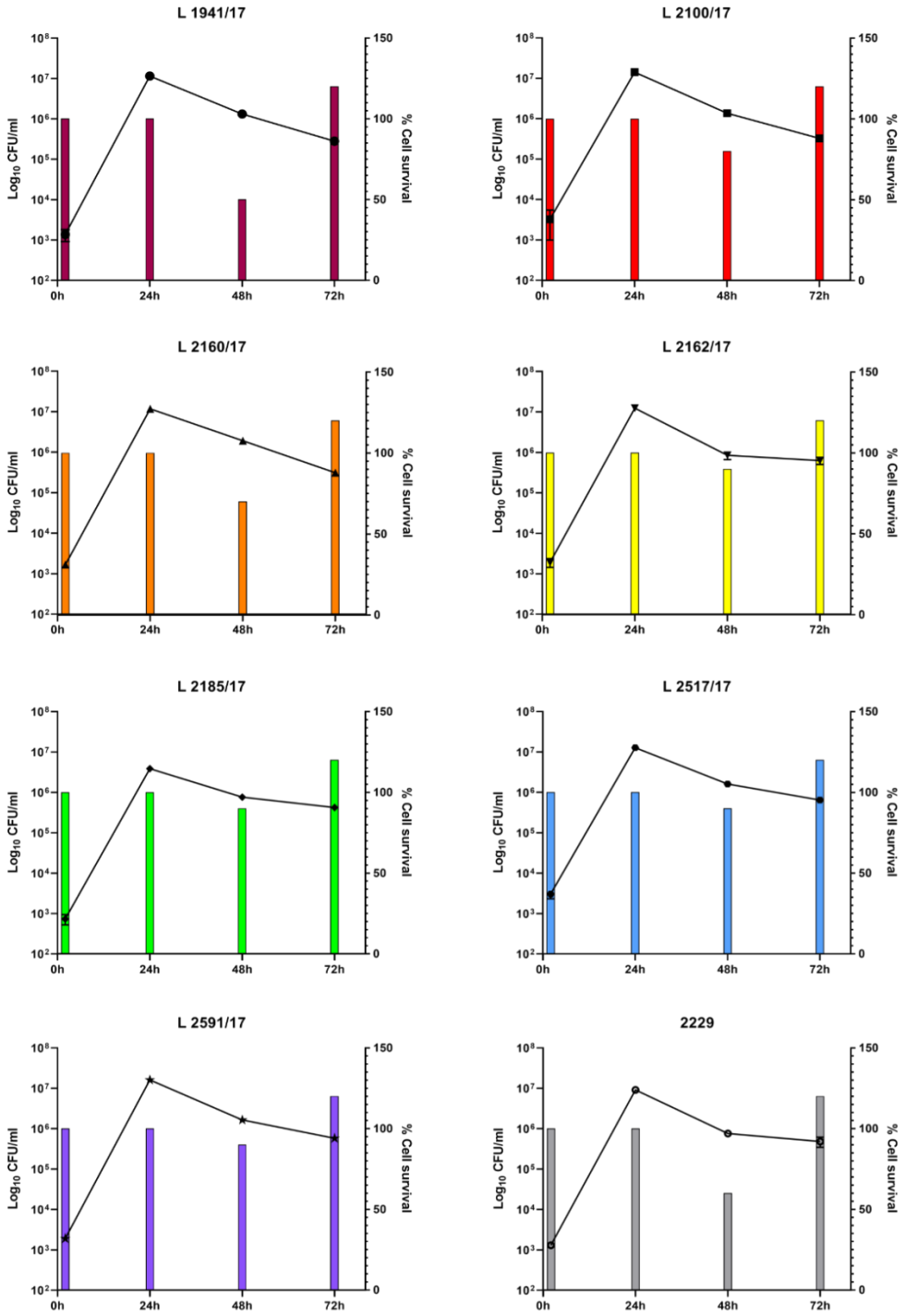


Figure 4.7 – Number of *S.* Dublin recovered at 2, 24, 48 and 72h alongside an estimated percentage survival of BCECs during infection study. *S.* Dublin was grown to log phase and diluted to a multiplicity of infection (MOI) of 1 to infect cultured bovine caruncular epithelial cells (BCECs) over the course of 72h. Cells were lysed at 2, 24, 48 and 72 hours, using isolates L 1941/17, L 2100/17, L 2160/17, L 2162/17, L 2185/17, L 2517/17, L 2591/1 and 2229, and cell survival was estimated at each time point. Bars refer to the percentage of cells surviving over time. Lines show the number of bacteria recovered from the infection studies. n=1

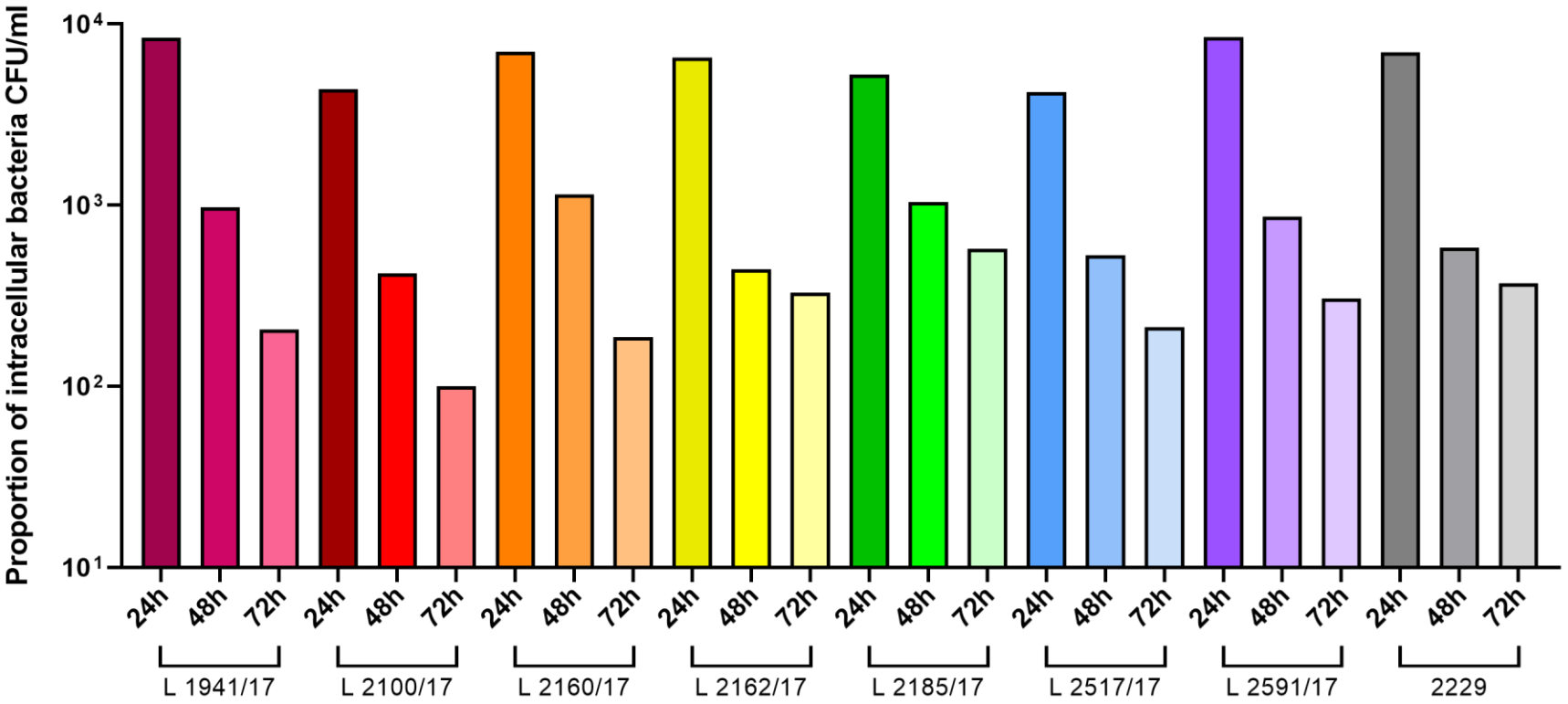


Figure 4.8 – Fold-change in *S.* Dublin recovered from infection of BCECs between 2h and 24, 48 or 72h post-infection. *S.* Dublin was grown to log phase and diluted to a multiplicity of infection (MOI) of 1 to infect cultured bovine caruncular epithelial cells (BCECs) over the course of 72h. Cells were lysed at 2, 24, 48 and 72 hours, using isolates L 1941/17, L 2100/17, L 2160/17, L 2162/17, L 2185/17, L 2517/17, L 2591/1 and 2229. Presumptive intracellular bacteria were sampled, serially diluted and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938). Fold change was calculated and presented for each time point. n=1

4.3.4 - Infection of BCECs with *S.* Dublin MOI of 1 and 10 over the course of 24h

To investigate the virulence of the different isolates in BCECs at a relatively low MOI and provide an insight into how these isolates interact with placental tissues in the host, an MOI of 1 was used over 24h. There was no significant variation between inocula between the isolates across the replicate experiments (Figure 4.9 A). All isolates were able to infect BCECs to the same degree, with $3.00x10^2$ to $9.07x10^3$ CFU/well recovered after 2h (Figure 4.9 B). After a 24h infection, all of the isolates were recovered at an average of $6.95x10^6$ CFU/ml (Figure 4.9 C).

Infections using an MOI of 10 demonstrates how a larger number of bacteria interact with the BCECs, whilst maintaining a consistent number of BCECs. The inocula for each experiment was consistent (Figure 4.10 A and Figure 4.11 A). All isolates were able to infect BCECs, with $2.57x10^3$ to $1.27x10^5$ CFU/well recovered after 2h (Figure 4.10 B). Isolates L 1941/17, L 2100/17, L 2104/17 and L 2135/17 showed a higher level of variation in the number of recovered bacteria at 2h in replicate experiments compared to the other isolates used (Figure 4.10 B). All of the isolates were recovered at an average of $8.74x10^6$ CFU/ml after 24 hours (Figure 4.11 B). The number of bacteria recovered after 24h did not vary between isolates across the replicate experiments.

There were no significant differences between the number of bacteria recovered after 24h independent of the inoculum MOI, despite seemingly striking differences in the fold-change between 2 and 24h for each MOI Figure 4.12.

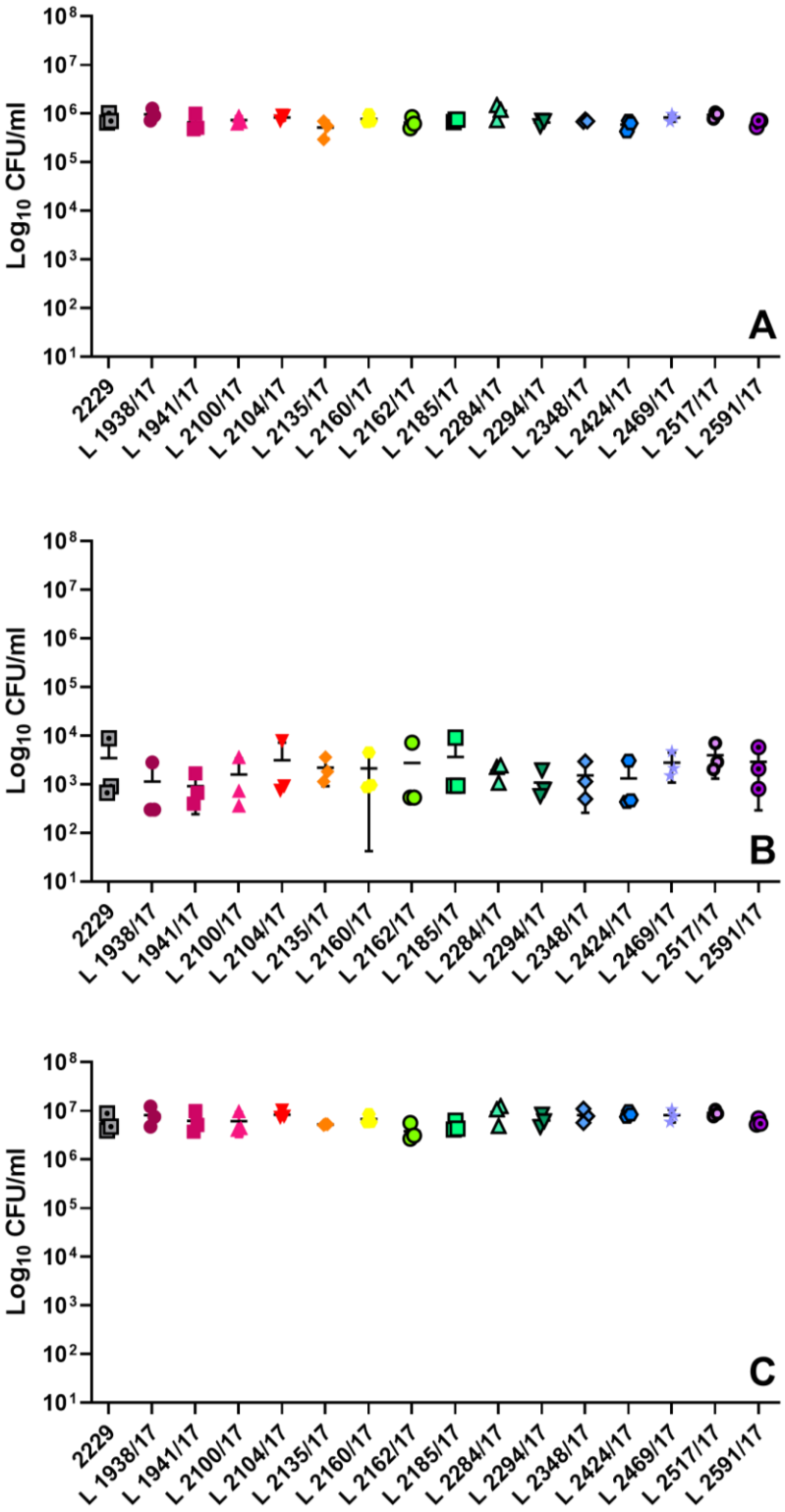


Figure 4.9 – *S.* Dublin infection study of BCECs using an MOI of 1. *S.* Dublin isolates were grown to log phase, diluted in bovine caruncular epithelial cell (BCEC) medium and used to inoculate BCECs at a multiplicity of infection (MOI) of 1 (A). The cells were cultured along with the bacteria and lysed at either 2 (B) or 24h (C) after infection. Bacteria were serially diluted and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938), and cultured overnight at 37°C, after which colonies were counted. n=3

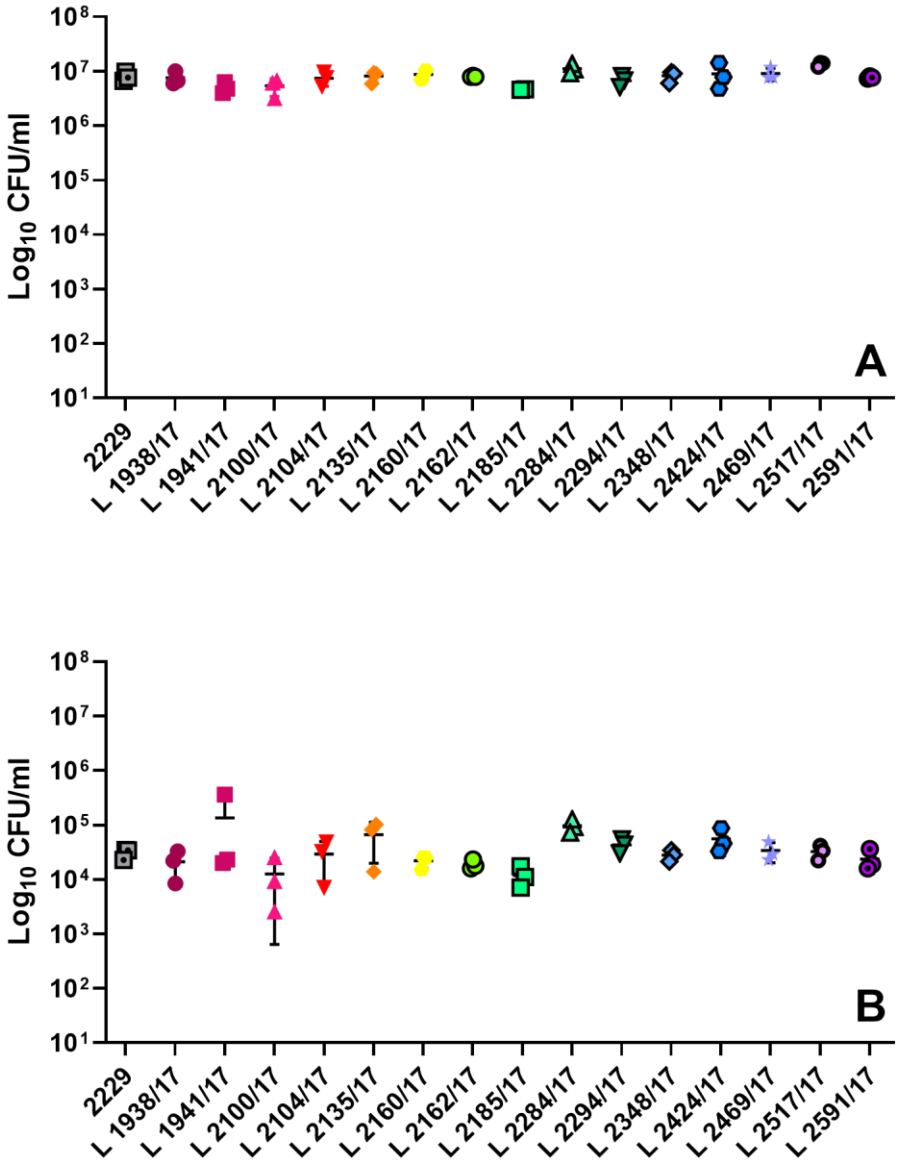


Figure 4.10 – *S.* Dublin 2h infection study of BCECs using an MOI of 10. *S.* Dublin isolates were grown to log phase, diluted in bovine caruncular epithelial cell (BCEC) medium and used to inoculate BCECs to a multiplicity of infection (MOI) of 10 (A). The cells were cultured along with the bacteria and lysed at 2h (B). Bacteria were serially diluted and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938), and cultured overnight at 37°C, after which colonies were counted. Data points for isolates L 2160/17 and L 2185/17 are absent due to colonies being uncountable (A). n=3

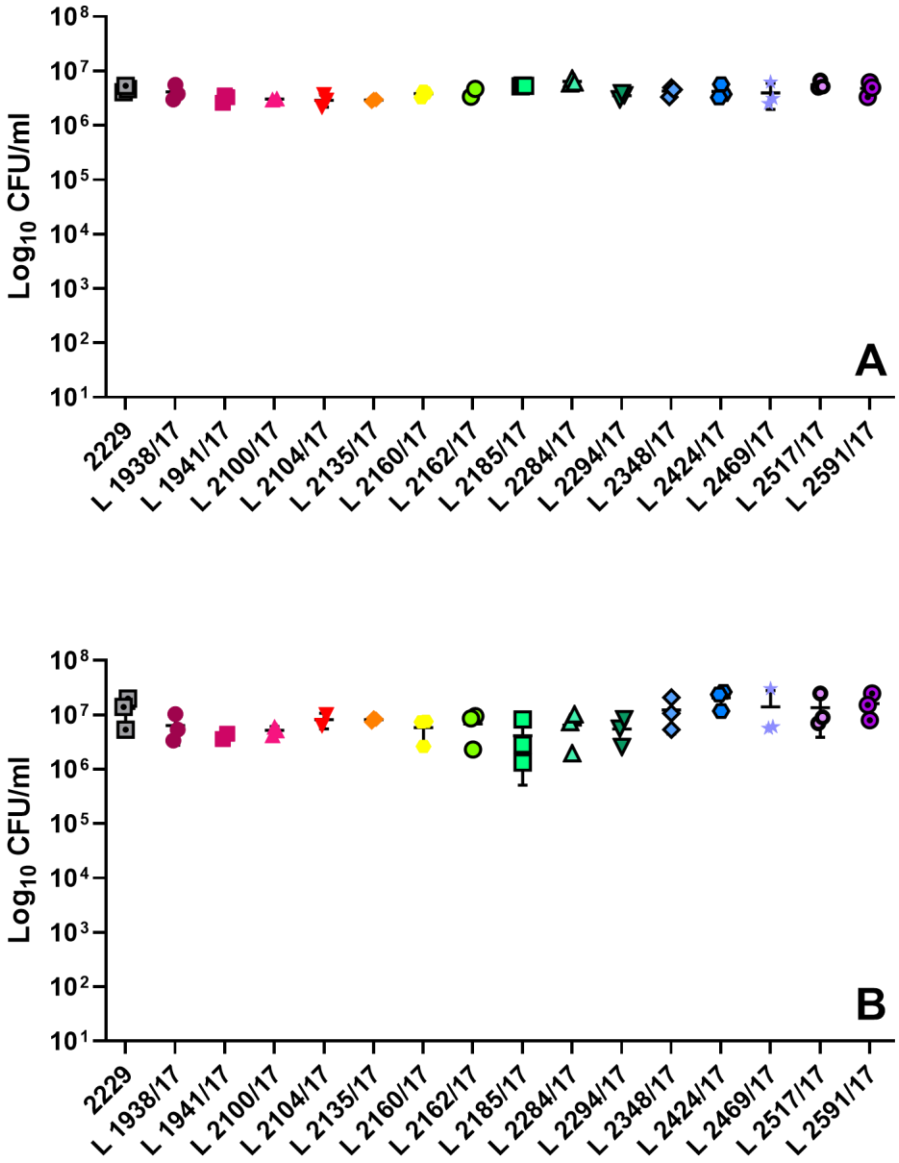


Figure 4.11 – *S.* Dublin 24h infection study of BCECs using an MOI of 10. *S.* Dublin isolates were grown into log phase and diluted in bovine caruncular epithelial cell (BCEC) medium and used to inoculate BCECs to a multiplicity of infection (MOI) of 10 (A). The cells were cultured along with the bacteria and lysed at 24h (B). Bacteria were serially diluted and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938), and cultured overnight at 37°C, after which colonies were counted. Data points for isolates L 2100/17 and L 2162/17 are absent due to the colonies being uncountable (A). n=3

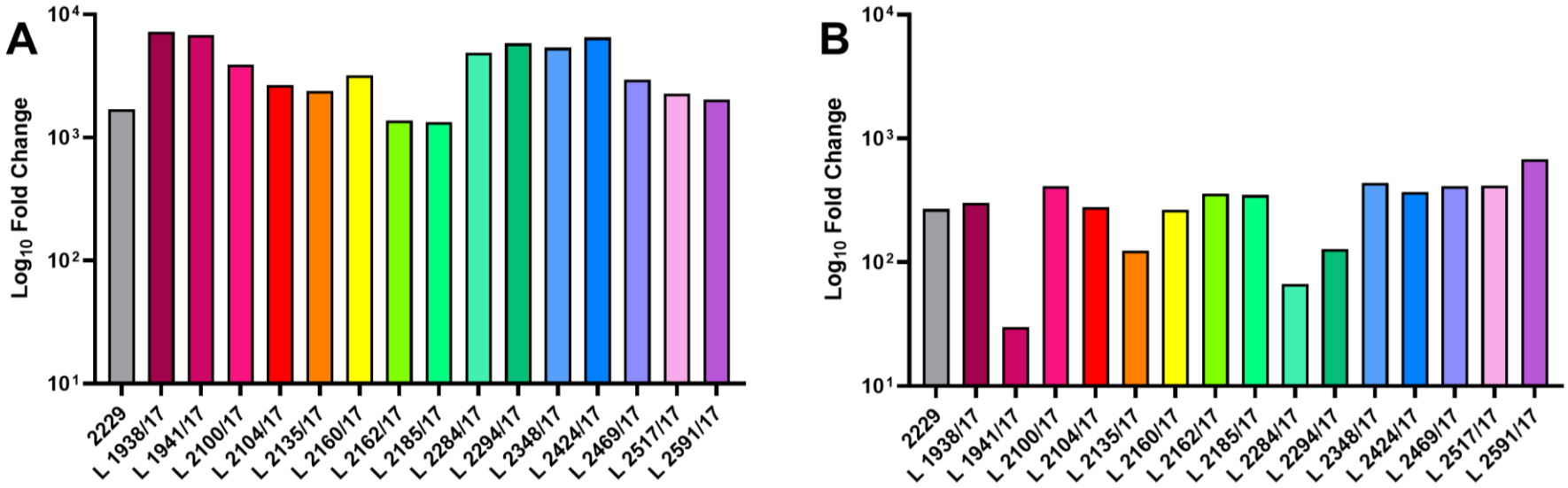


Figure 4.12 - Fold change of *S.* Dublin recovered from infection of BCECs between 2 and 24h post-infection at MOIs of 1 (A) and 10 (B). Bovine Caruncular Epithelial Cells (BCECs) were infected with *S.* Dublin isolates from livestock sources at multiplicities of infection (MOIs) of 1 and 10. BCECs were lysed after 2 and 24h and the presumptive intracellular bacteria were sampled, serially diluted and plated onto nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938). The fold change between the bacteria recovered after 2h of infection and after 24h of infection was calculated and presented. n=3

4.3.5 - Pilot Study – Inoculation of whole bovine blood over the course of 24h

Isolates representing various elements of interest previously identified were used to infect fresh bovine whole blood over the course of 24h. The pilot study was used to determine whether the chosen time intervals were appropriate in this investigation.

It was not possible to determine the initial inoculum of this experiment, however a consistent decrease in CFU/ml was observed across the four isolates over the first 90 minutes and numbers remained relatively constant for the following time points (Figure 4.13). 24 hours post-infection, there was an increase in bacterial count compared to 300 min post infection which varied between isolates (Figure 4.13).

Based on these results, the sampling regime was amended to 5-minutes post inoculation and at 10-minute intervals post inoculation for the first 60 minutes, then at 90 and 120 minutes.

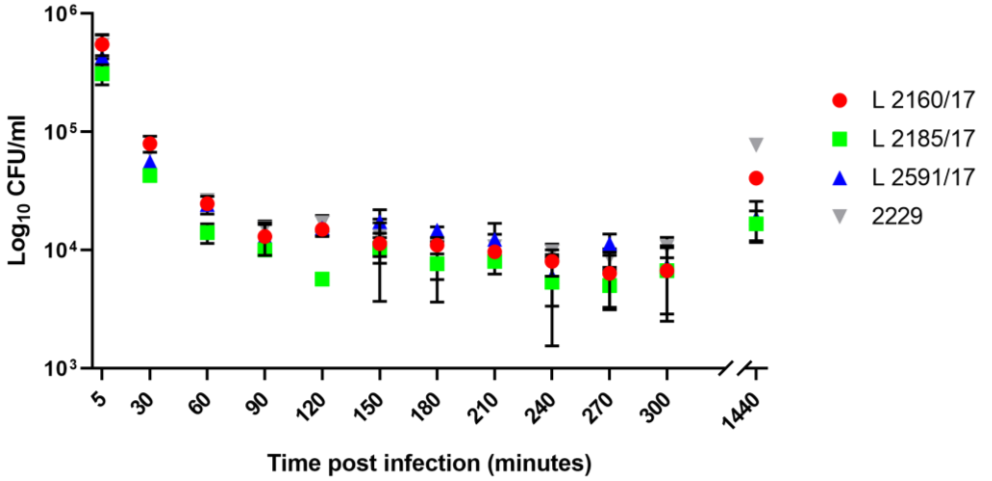


Figure 4.13 - Pilot study of *S.* Dublin infection of fresh whole bovine blood over the course of 24 hours. Blood was collected 30 minutes prior to infection from cattle not receiving antibiotic treatment and deemed healthy by a veterinarian. Blood was aliquoted into 15ml tubes and incubated on a roller in 5% CO_2 and at 37°C. *S.* Dublin isolates were grown into log phase in nutrient broth and diluted to approximately $1x10^6$ CFU/ml in each of the blood tubes. The tubes were incubated in conditions previously stated over the course of 24h, with samples being taken intermittently. Samples were serially diluted and plated on nutrient agar, according

to the Miles and Misra method (Miles, Misra, and Irwin, 1938). Plates were incubated at 37°C overnight and colonies were counted. n=1

4.3.6 - Survival of *Salmonella* Dublin in fresh bovine whole blood

The survival of *S.* Dublin in whole blood was investigated over the course of 2h of infection. Investigation the survival of *S.* Dublin in fresh bovine whole blood may allow for identification of differences between isolates that may impact on dissemination in the blood during infection.

A characteristic pattern of reducing CFU/ml over the first 90 minutes was observed in all isolates and in all experimental repeats (Figure 4.14). The number of bacteria in whole blood was significantly reduced in isolates L 2160/17, L 2185/17 and L 2591/17 after 90 minutes ($P<0.05$), and all isolates were significantly reduced after 120 minutes of infection ($P<0.05$) (Figure 4.14). The numbers of bacteria reduced between 63 and 83-fold compared to the inoculum after 30 minutes for isolates L 2160/17, L 2185/17 and L 2591/17, and between 65 and 102-fold after 60 minutes in all isolates (Table 4.5). The survival of the bacteria over the first hour did not vary between isolates, nor did the rate of bacterial cell death in the first 60 minutes (Table 4.6).

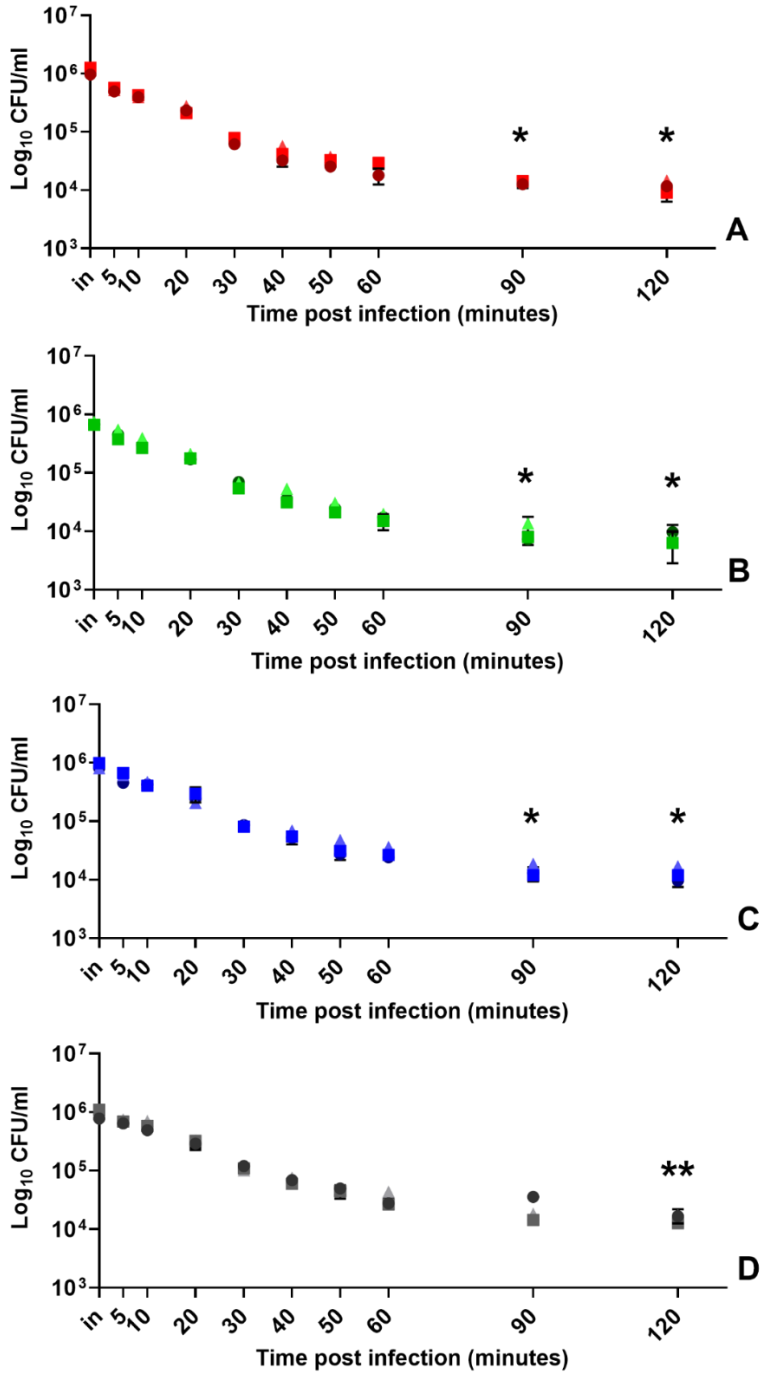


Figure 4.14 - Survival of *S.* Dublin isolates in fresh whole bovine blood over the course of 24h. Blood was collected 30 minutes prior to infection from cattle not receiving antibiotic treatment and deemed healthy by a veterinarian. Blood was aliquoted into 15ml tubes and incubated on a roller in 5% CO_2 and at 37°C. *S.* Dublin isolates were grown into log phase in nutrient broth and diluted to approximately $1x10^6$ CFU/ml in each of the blood tubes. The blood tubes were incubated in conditions previously stated for 2h, with samples being take intermittently. Samples were serially diluted and plated on nutrient agar, according to the Miles and Misra method (Miles, Misra, and Irwin, 1938). Plates were incubated at 37°C overnight and colonies were counted. A isolate L 2160/17. B isolate L 2185/17. C isolate L 2591/17. D isolate 2229. * = $P<0.05$; ** = $P<0.005$. n=3.

Table 4.5 – Fold-change in CFU/ml of *S.* Dublin isolates compared to the inoculum at different time points post-inoculation in whole bovine blood studies.

Isolate	Fold-reduction in CFU/ml post-inoculation (minutes)			
	30	60	90	120
L 2160/17	16	46	83*	102*
L 2185/17	11	40	76*	90*
L 2591/17	10	31	63*	71*
2229	9	31	52	65**

Fresh whole bovine blood was collected 30 minutes prior to infection from cattle not receiving antibiotic treatment and deemed healthy by a veterinarian. Blood was aliquoted into 15ml tubes and incubated on a roller in 5% CO_2 and at 37°C. *S.* Dublin isolates were grown into log phase in nutrient broth and diluted to approximately $1x10^6$ CFU/ml in each of the blood tubes. The tubes were incubated in conditions previously stated over the course of 2 hours with samples being taken intermittently. Samples were serially diluted and plated on nutrient agar, according to the Miles and Misra method (Miles, Misra, and Irwin, 1938). Plates were incubated at 37°C overnight and colonies were counted. Asterisk denotes a statistically significant reduction in CFU/ml (Kruskal-Wallis and Dunns Multiple Comparisons. * = $P<0.05$; ** = $P<0.005$). n=3

Table 4.6 - Rate of *S.* Dublin death in whole bovine blood over 60 minutes.

Isolate	Rate of *S.* Dublin death in the first 60 minutes (min^{-1}) ±SD	
L 2160/17	-0.03756	± 8.85E-03
L 2185/17	-0.05839	± 8.90E-03
L 2591/17	-0.05644667	± 6.95E-03
2229	-0.05785667	± 2.56E-03

Fresh whole bovine blood was collected 30 minutes prior to infection from cattle not receiving antibiotic treatment and deemed healthy by a veterinarian. Blood was aliquoted into 15ml tubes and incubated on a roller in 5% CO_2 and at 37°C. *S.* Dublin isolates were grown into log phase in nutrient broth and diluted to approximately $1x10^6$ CFU/ml in each of the blood tubes. The tubes were incubated in conditions previously stated over the course of 2 hours with samples being taken intermittently. Samples were serially diluted and plated on nutrient agar, according to the Miles and Misra method (Miles, Misra, and Irwin, 1938). Plates were incubated at 37°C overnight and colonies were counted. A logistic growth curve was fitted to CFU/ml multiplied by -1, using GraphPad Prism and the rate of bacterial cell death was obtained for the first 60 minutes of the experiment. n=3

4.4 - Discussion

To aid in understanding the pathogenesis of *S.* Dublin infection leading to abortion, the virulence of sixteen isolates was investigated in a monoculture model of the placentome. The pilot studies investigating different MOIs and timelines for infection studies demonstrated that an MOI of 1 and 10 over the course of 24h would be the most appropriate conditions for future experiments, similar to other studies using BCECs (Haeger et al., 2015; Jiménez-Pelayo et al., 2019a).

All *S.* Dublin isolates were recovered to an extent from the infection studies, so it is assumed that the bacteria were intracellular and protected from the gentamycin (Elsinghorst, 1994). However, this inference requires experimental validation, potentially through the use of microscopy and GFP-producing *S.* Dublin isolates to visualise the bacteria within a cell.

All *S.* Dublin isolates were able to invade and replicate within BCECs to similar extents over the course of 24h, inferred by their recovery from gentamycin protection-like assays. This was to be expected as all of the isolates used in this study were isolated from clinical cases of disease and therefore must have infected bovine tissues in order to cause disease. Furthermore, as demonstrated previously by genotypic characterisation, all of the isolates were identical in their SPI-1 genes, the pathogenicity island primarily responsible for the invasion of mammalian cells, including epithelial cells (Velge et al., 2012). According to the VFDB-inferred results previously obtained, isolate L 2160/17 differed from the other fifteen isolates in that the SPI-2 gene *ssaU* was of less than 90% similarity, indicating that this isolate may differ in its capability for intracellular survival (Yu et al., 2018). However, the heterologous *ssaU* sequence of this isolate did not appear to impact its survival in BCECs over the course of 24h. This too was to be expected as SPI-2 is usually associated with survival in macrophages rather than epithelial cells and is induced via

the acidic pH of these cells due to the presence of reactive oxygen and nitrogen species (Rappl, Deiwick, and Hensel, 2003).

The recovery of similar numbers of isolate L 2185/17 from BCECs compared to all other isolates was unanticipated, as this isolate was previously found to have a biphasic growth pattern which was lower compared to that of the other isolates. Invasion of host cells by *Salmonellae* involves the formation of the *Salmonella*-containing vacuole (SCV) via bacteria-induced endocytosis localised to specific regions within the host cell (LaRock, Chaudhary, and Miller, 2015; Ramsden, Holden, and Mota, 2007). The observation that isolate L 2185/17 is recovered in similar numbers to the other isolates despite this difference in growth, implies that there may be an upper threshold to the number of intracellular bacteria that can reside within the SCV. Alternatively, the growth rate of the bacterium is different inside the SCV compared to in media. This is corroborated by the fact that similar numbers of bacteria for all isolates are recovered regardless of initial MOI. A potential explanation for this may be that carbon availability is limited within the SCV, so a certain number of *Salmonellae* can replicate within the SCV before this hinders intracellular replication. *Salmonellae* are also capable of cytosolic replication upon exit of the SCV, so it is also possible that these isolates were limited in their capacity for intracellular replication in the cytosol rather than the SCV. It is not possible to determine which in this work, although cytosolic "hyper-replication" in *S.* Typhimurium is associated with epithelial cell death and release of invasion-primed bacteria, potentially facilitating rapid bacterial spread (Knodler et al., 2010). In order to investigate this further, fluorescently labelled *S.* Dublin isolates could be used to infect BCECs and single-cell flow cytometry could help to discern intracellular localisation and bacterial numbers. This would also validate the inference that the *S.* Dublin isolates were intracellular during infection of BCECs.

Whilst some variation between isolates was observed in previous studies, this variation was not linked to epithelial cell invasion or

intracellular replication in BCECs, indicating that these differences may not produce contrasting characteristics in clinical outcome. As previously indicated in similar work investigating *S.* Dublin, it may be more likely that variation in clinical outcome is a result of differing host immune responses rather than divergence among bacteria (Mohammed et al., 2017).

Investigating the ability of the bacteria to survive in whole bovine blood gives an understanding of how the bacteria might survive in this complex host environment once they have traversed the intestinal barrier. Whilst *Salmonellae* are known to be able to infect and persist within phagocytes and in cell-free niches in the lymphatic system, there is little evidence that these hypotheses lead to translocation of the bacterium to the placentome (Pullinger et al., 2007). Additionally, the possibility of *S.* Dublin being disseminated in the blood freely has not previously been explored.

A consistent pattern of reduced bacterial viability was observed over 2 hours, demonstrating the bactericidal activity of bovine blood against *S.* Dublin. It is also possible to identify fundamental differences between bacterial virulence using whole blood infection studies, demonstrated by investigation of the impact of Vi capsular antigen on *S.* Typhi growth in human blood and the variation between host adapted *S.* Gallinarum and host generalist *S.* Enteritidis in chicken blood (Liaquat et al., 2015; Sreekantapuram et al., 2021). Given the high level of similarity between the isolates used in the study described here, it was expected that there would be a consistent reduction of bacterial viability with little intra-isolate variation. Large differences in different studies were usually observed when differences between isolates were either far greater (as in the case of observing different serovars) or the small differences were more likely to have a larger impact on blood survival (in the case of Vi capsular antigen) (Liaquat et al., 2015; Sreekantapuram et al., 2021). These similarities demonstrated that the apparent heterologous *ssaU*

sequence in isolate L 2160/17 is unlikely to be of great importance in the blood as all isolates were highly similar.

Similar to other studies, a small percentage of bacteria were recovered compared to the controls after 2h (Allert et al., 2022; Kämmer et al., 2020). This may provide evidence that a small number of *S.* Dublin are able to survive in the blood, and their survival over the course of two hours indicates the potential for dissemination away from the gastrointestinal tract. Infection with *S.* Dublin is reported to cause bacteraemia in both humans and cattle (Dias et al., 2009; Laupland et al., 2010; Nielsen, 2013b). *S.* Dublin has been detected in jugular blood in experimentally infected cattle from 2h post infection, but no other experiments into the impact of free-living *S.* Dublin in the blood of cattle has been conducted (Pullinger et al., 2007). More work is required to evaluate the hypothesis that the dissemination of free-living bacteria could be part of the pathogenesis of abortions in cattle due to *S.* Dublin.

The BCEC study was limited by the fact that this is a simple single cell model when the placentome is a highly complex structure made up of different tissues of both fetal and maternal origin. Additionally, this cell line was isolated from a single animal, meaning intra-species differences cannot be accounted for in this model. The experimental design also deliberately avoided using a bacterial MOI which led to significant cell death. These experiments show that *S.* Dublin can infect these reproductive cells as proof of concept, but it would be useful to see the progression of infection from the maternal tissues to the fetal tissues and observe any tissue destruction. Fluorescently labelled *S.* Dublin isolates could also be used so that they could be easily identified in the different tissues and structures in the placenta during infection over time. The whole blood study was a much more complex environment in which to investigate bacterial survival, but this is still limited as normal circulation would bring about more phagocytic cells and complement to facilitate bacterial clearance. However, as these studies have been previously used, they serve as

a foundation upon which more extensive studies can build. It might be useful to use blood from pregnant animals and compare this to that of lactating animals in order to discern whether bacteria are more capable of survival due to the hormonal states established during pregnancy. In both whole blood and BCEC infection, the use of transcriptomics could help identify genes upregulated or downregulated in these environments necessary to facilitate infection (Graham et al., 2005; Mereghetti et al., 2008; Toledo-Arana et al., 2009). This could then be used to identify therapeutic targets for future interventions.

4.5 - Summary

All abortion-associated isolates and reference isolate 2229 were able to invade and replicate within the BCECs over the course of 24h. All isolates invaded to the same degree and were able to survive in the BCECs to the same degree over 24h. Following from this work, it is possible to reduce the number of isolates in future, more labour-intensive studies, without introducing variability of bacterial virulence in BCECs. In whole blood, a limited panel of isolates survived to the same degree over the course of 2h which provides evidence of another potential route of dissemination, alternative to monocyte or macrophage carriage.

Whilst this work gives an insight into how *S.* Dublin interacts with the host, it does not demonstrate how the host may respond to infection. Therefore, BCECs will be utilised in infection studies like those presented here with a selection of *S.* Dublin isolates as a model to investigate how the host responds to infection.

Chapter 5 - Investigating the host response to *Salmonella* Dublin infection in Bovine Caruncular Epithelial Cell model

5.1 - Introduction

Despite *S.* Dublin being one of the most common causes of bovine abortion in the UK, very little is known about how and why infection leads to abortion. During infection, toll-like receptors (TLRs) would be triggered by *S.* Dublin ligands like LPS and outer membrane proteins, initiating downstream activation of pro-inflammatory response pathways which lead to the secretion of cytokines, chemokines and lipid signalling molecules. Examples of these inflammatory mediators include TNFα, CXCL8 and PGE_2 respectively.

TNFα is secreted by a variety of cell types and stimulates the immune system as well as limiting pathogen spread by initiating of apoptosis and forming lesions (Dealtry, O-Farrell, and Fernandez, 2000; Fair, 2015; Gohin et al., 1997; Gorivodsky et al., 1998; Liu et al., 2017; Mastroeni, Skepper, and Hormaeche, 1995; Roy and Malo, 2002; Vázquez-Torres et al., 2001). TNFα is thought to have beneficial actions during pregnancy, where tissue remodelling is crucial for fetal development (Sousa et al., 2021). Secretion of TNFα is initiated via TLR4 signalling, of which *Salmonella* LPS and a variety of other outer membrane molecules including porins are ligands (Cervantes-Barragán et al., 2009) CXCL8 is a chemoattractant for neutrophils, the most plentiful leukocytes in the blood (Rydell-Törmänen, Uller, and Erjefält, 2006; Saffarzadeh et al., 2012). CXCL8 primes neutrophils and other cells for bactericidal action and is essential for pathogen clearance in *Salmonella* infection (Oliveira et al., 2015). Like TNFα, CXCL8 has beneficial actions during pregnancy including

cervical ripening (Van Engelen et al., 2009). Pro-inflammatory responses without strict regulation leads to disease, so anti-inflammatory or immunoregulatory mechanisms are activated alongside pro-inflammatory responses. This includes the production of PGE_2, a lipid hormone with key immunoregulatory effects including inducing production of anti-inflammatory IL-10 and reducing the bactericidal action of macrophages through downregulation of radical production (Liu et al., 2012; Stolina et al., 2000). The inflammatory process initiated in response to *S.* Dublin, including the mediators described here, would usually contribute to pathogen clearance whilst preventing host tissue damage through inappropriate inflammation. However, the inflammatory process in response to *S.* Dublin during pregnancy could differ substantially and lead to abortion.

In ungulates, an up-regulated Th1 response is thought to be part of the pathogenesis of abortifacient infections like *Neospora caninum, Listeria monocytogenes* and *Trueperella pyogenes* in cattle and *Chlamydia abortus* in sheep (Barber, Fazzari, and Pollard, 2005; Borges, Healey, and Sheldon, 2012; Entrican, Buxton, and Longbottom, 2001; Quinn, Ellis, and Smith, 2002). The host response to *S*. Dublin in the bovine reproductive tract has been evaluated in an explant model of the endometrium and placenta but inactivated bacteria were used, preventing the investigation of the immunomodulatory actions of *Salmonellae* (Silva et al., 2012). Therefore, only speculations based on other reproductive diseases and *Salmonella* in non-specific tissues or as inactivated TLR ligands can be made as to how *S.* Dublin leads to an abortion event in cattle. This work is the first to use a host and tissue specific model to explore the immune response to infection as part of the pathogenesis leading to abortion in cattle due to *S.* Dublin.

5.1.1 - Aims and hypotheses

This chapter aims to characterise the BCEC immune response to previously characterised *S.* Dublin isolates using CXCL8, TNFα as pro-inflammatory and PGE_2 as immunomodulator markers of the host

response. This includes identifying suitable reference genes in the *S.* Dublin-infected BCEC model for use in qPCR.

We hypothesise that cells stimulated with LPS from *S.* Typhimurium and heat killed *S.* Dublin and infected with live *S.* Dublin isolates will express elevated levels of CXCL8, TNFα and PGE_2 compared to unstimulated and uninfected controls.

5.2 - Materials and Methods

5.2.1 - Inocula containing BCEC stimulants

BCECs were infected using four *S.* Dublin isolates identified in previous studies at MOIs of 1 and 10 and stimulated using 1µg/ml lipopolysaccharide (LPS) from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich) and heat-killed *S.* Dublin isolate 2229 at MOIs of 1 and 10 (Table 5.1). Culturing of *S.* Dublin isolates and production of an inoculum at different MOIs was previously described (4.2.2 - Bacterial culturing and producing inoculum).

To produce the heat-killed inoculum, 3 colonies of isolate 2229 were picked and cultured overnight in 5ml of sterile NB at 37°C in an orbital shaking incubator. The following day, 1ml of this culture was transferred into 4ml of sterile NB and incubated at 37°C in an orbital shaking incubator for 2 hours. Based on previous growth studies, this culture was estimated to be $1.02x10^9$ CFU/ml (±1.73CFU/ml). 1ml of this culture was placed into a 1.5ml tube and incubated at 100°C for 10 minutes. 20µl of the heat-killed sample was placed onto NA and incubated overnight at 37°C to ensure that the sample contained no live bacteria. Inocula were produced by diluting the heat-killed sample in 1ml pre-warmed antibiotic-free BCEC media in the same ratio as if the sample contained live bacteria.

Table 5.1 - Different stimulation regimen of BCECs.

Stimulant	Detail
L 2160/17 (2160)	*ssaU* <90% identity; Clade 2 in WGS alignment; clusters closely with majority of other livestock isolates in accessory alignment
L 2185/17 (2185)	Biphasic growth pattern; Clade 3 in WGS alignment; clusters closely with majority of other livestock isolates in accessory alignment
L 2591/17 (2591)	Clade 1 in original analysis; most distant clustering in accessory alignment from other test isolates
2229 (2229)	Reference isolate from calf diarrhoea; Clade 3 in WGS alignment; clusters with smaller island of isolates in accessory alignment
Heat-killed 2229 (HK29)	Equivalent concentrations to MOIs of live bacteria of 1 and 10
1µg/ml LPS (LPS)	From *Salmonella enterica* serovar Typhimurium

Live and heat-killed bacteria were used to stimulate cells at multiplicities of infection (MOI) of 1 and 10. Isolate 2229 at MOIs of 1 and 10 were incubated at 100°C for 10 minutes to produce heat-killed isolate 2229. Bovine caruncular epithelial cells (BCECs) were stimulated for 1h and incubated for a total of 2 or 24h before being lysed. A total of 11 different stimulation regimen were used.

5.2.2 - Stimulation of BCECs

BCECs were cultured as previously described until confluent in T75 flasks, approximately $8.4x10^6$ cells (Table 5.2). 24 hours before use, cells were passaged into 6 well plates with approximately $1.2x10^6$ cells per well. 1h before stimulation, media was aspirated and replaced with 1ml pre-warmed antibiotic-free BCEC medium and incubated again at 37°C for 1 hour. Antibiotic-free BCEC medium was aspirated from the wells, and inocula containing live bacteria at MOIs of 1 and 10, heat-killed bacteria or LPS was added (Table 5.1). LPS was used at a concentration of 1µg/ml, mirroring the work of others investigating the host response to different immune challenges in different cell types in cattle (Cronin et al., 2012; Jungi et al., 1996; Zhang et al., 2019). The stimulated cells were incubated at 37°C and 5% CO_2. After 1 hour, the medium was removed, and replaced with 1ml of cell culture medium containing 100µg/ml gentamycin (Fisher Scientific UK Ltd) and incubated for 1 hour at 37°C and 5% CO_2 to kill any extracellular bacteria (Elsinghorst, 1994). For a 2h stimulation,

approximately 1ml of the media was removed and frozen at -80°C. Cells were lysed using 350µl of Buffer LBP (NucleoSpin RNA Plus isolation kit, Macherey-Nagel). For a 24h stimulation, the media was removed and replaced with 1ml BCEC culture media containing 5µg/ml gentamycin. These cultures were incubated for a further 22h. Collection of culture medium, cell lysis, collection of lysate and storage of samples was carried out as for 2h stimulations. Lysate was stored at -20°C prior to RNA isolation.

Table 5.2 – Passage numbers and time in culture of BCECs used in each experimental set of stimulations.

Experimental set	Cell passage number	Time in culture (days)
A	40	43
B	40	43
C	32	25
D	32	16
E	35	18

Bovine caruncular epithelial cells (BCECs) were cultured and passaged to be infected with *S.* Dublin isolates to discern the host response to infection.

5.2.3 - RNA isolation

RNA isolation was completed using the NucleoSpin RNA Plus isolation kit (Macherey-Nagel) according to the manufacturers' instructions. Briefly, the cell lysate was thawed and transferred to a NucleoSpin gDNA Removal Column placed in a collection tube, and centrifuged for 30 seconds at 11,000*g*. The Column was discarded and flow-through retained. 100µl of Binding Solution BS was added to the flow-through and pulse-vortexed for 5 seconds. The total lysate (450µl) was transferred to a NucleoSpin RNA Plus Column placed in a collection tube. This was centrifuged for 15 seconds at 11,000*g*. 200µl of Buffer WB1 was added to the column and centrifuged for 15 seconds at 11,000*g*. The collection tube and flow-through were discarded. The column was retained and placed into a new collection tube. 600µl Buffer WB2 was added to the column and centrifuged for 15 seconds at 11,000*g*. The flow-through was discarded and column retained and placed back into the collection tube. 250µl Buffer WB2

was added to the column and centrifuged for 2 minutes at 11,000*g* to completely dry the membrane. The column was placed into a nuclease-free collection tube. 30µl of RNase-free H_2O was added to the collection tube and centrifuged at 11,000*g* for 1 minute. A further 30µl of RNase-free H2O was added to the collection tube and centrifuged at 11,000*g* for 1 minute.

5.2.4 - RNA Quantification

RNA was quantified using Qubit HS/BR RNA Assays according to the manufacturers' instructions (Invitrogen). A Working Solution of 200µl of Qubit HS/BR Buffer (Invitrogen) and 1µl of Qubit HS/BR Reagent (Invitrogen) was prepared for each sample. The Working Solution was vortexed for 3 seconds to ensure homogeneity. For Standardisation, Qubit HS/BR Standards 1 and 2 (Invitrogen) were added into 190µl of Working Solution each in Qubit Assay Tubes (Invitrogen). The Standards were then vortexed for 3 seconds and incubated at room temperature for 2 minutes. The Standards were then read in the Qubit Fluorometer.

For each sample, 198µl of Working Solution was aliquoted into Qubit Assay Tubes and 2µl of each sample was added. The tubes were vortexed for 3 seconds and incubated at room temperature for 2 minutes. The samples were then read in the Qubit Fluorometer.

5.2.5 - PCR

Prior to cDNA synthesis, RNA samples were assayed for genomic DNA contamination using a PCR targeting GAPDH. The presence of a band indicated the presence of DNA and therefore contamination. 2µl of each RNA sample was added to a master mix consisting of 25µl DreamTaq Green Master Mix 2x (Thermo Fisher Scientific), 5µl forward and reverse primers (with a final concentration of 1µM) and 13µl molecular grade water (Thermo Fisher Scientific) for a total reaction volume of 50µl. cDNA at 1ug/ml from a previous experiment was used as a positive control and 1ul sterile molecular grade water was used as a negative control. Cycling conditions consisted of an

initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, plus a final extension step at 72°C for 10 minutes. The size of PCR products was confirmed by gel electrophoresis, run on a 2% agarose gel at 90V for 1h40m before being visualised.

After cDNA was synthesised, a standard PCR for was performed to confirm the presence of cDNA. The same PCR protocol was followed as previously described, including positive and negative controls previously used.

Standard PCR was also used to confirm the identity of products produced using qPCR primers for ACTB, C2orf29, GAPDH, SUZ12, TBP, CXCL8 and TNFα using the qPCR primers at their optimised annealing temperatures (Table 5.3) and the standard PCR master mix previously described. SYBR Green is an intercalating agent which means any non-specific binding of primers during the annealing process would contribute to the fluorescent signal during qPCR. A gel of 2% agarose was used in a 1h 40m electrophoresis to visualise the band sizes against a 100bp ladder and confirm the bands were the predicted size fractions (Appendix Figure 9.11). The DNA products were quantified as previously described (section heading) and underwent Sanger Sequencing by Eurofins Scientific. The sequencing result from each product was input into NCBI BLAST to confirm product identity (Altschul et al., 1990).

5.2.6 - DNA digest and extraction of contaminated RNA samples

RNA samples found to be contaminated with genomic DNA underwent a "clean-up" procedure before being used to produce cDNA. rDNase (Macherey-Nagel) and Reaction Buffer (Macherey-Nagel) were mixed in a ratio of 1:10 to make an Enzyme-Buffer Mix. 1 part of this Enzyme Buffer Mix was then added to 9 parts RNA sample and incubated for 10 minutes at 37°C. The RNA was purified by adding RNA extraction buffer LBP (Macherey-Nagel) to make a total volume of 350µl before

completing the extraction process as previously described. The RNA was quantified using the Qubit procedure previously described as well as undergoing a GAPDH PCR to check for residual genomic DNA contamination as previously described.

5.2.7 - cDNA synthesis

Once RNA was confirmed to be free of genomic DNA, a sample was taken and diluted in sterile molecular-grade water (Sigma-Aldrich) to produce a final concentration of 0.1ug/µl in a volume of 14µl. 1ul of Random Primers (Promega) were added to each sample for a final volume of 15µl and incubated for five minutes at 70°C before being transferred directly onto ice. 1µl Moloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase (Promega) was added to 5µl 5x MMLV Buffer (Promega), 0.5µl RNase Inhibitor (Promega), 1.25µl dNTPs (New England Biolabs) and 2.25µl sterile molecular grade water (Sigma-Aldrich) for a final volume of 25µl and incubated at 21°C for ten minutes and at 42°C for one hour. A negative control was made in each batch of cDNA produced by adding all reagents except MMLV Reverse Transcriptase and replacing this with 1µl sterile molecular grade water.

5.2.8 - qPCR

Expression of bovine reference genes and cytokines mRNA was quantified using the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad). 5µl 1µg/ml cDNA was added to a 15µl Master Mix (Table 5.4) for a total reaction volume of 20µl. Cycling conditions of initial denaturation step of 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 15 seconds at an optimised annealing temperature (Table 5.3) and 72°C for 10 seconds were used. All reactions including standards, no template controls (NTCs, consisting of Master Mix without cDNA) and samples were carried out in triplicate on each qPCR plate. A melt curve was included in each experiment with a single peak denoting single product amplification. NTCs producing fluorescent signals for ACTB and SUZ12 were investigated by identifying their melt temperature on the melt curve and using

electrophoresis on a 2% agarose gel to estimate the band size. This confirmed that the signal was caused by a primer dimer rather than an alternative qPCR product.

qPCR data were analysed using GraphPad Prism. Standard deviation of the cycle threshold (Ct) in triplicate reactions was calculated in Microsoft Excel. If this exceeded 1.10 (plus 10%), the samples or standards were repeated. A linear regression was performed on the Ct values for standards to obtain the slope value and R^2 for primer efficiency and pipetting accuracy respectively. Efficiency was calculated using the slope value obtained from the linear regression (Equation 5.1).

$$E\% = 10^{\frac{-1}{slope}} x100$$

Equation 5.1 – Equation to calculate qPCR efficiency.
E% = qPCR efficiency
Slope = slope value obtained from plotting linear regression of Ct values from qPCR standard curve.

5.2.8.1 - Assessment of reference gene stability using GeNorm

geNorm (part of the qBase+ software package) was used to assess the stability of the five reference genes identified (Vandesompele et al., 2002). The genes with the lowest *M* values (corresponding to gene stability) were chosen for normalisation.

5.2.8.2 - Calculation of normalised target gene expression

To obtain the relative expression of a target gene, a differentiation factor was first calculated based on the expression of a reference gene (Equation 5.2) and used to normalise the expression of the target genes, both in relation to the slope values of the standards obtained with each experimental run (Equation 5.3) (Hughes et al., 2007). The normalised expression of a target gene was calculated using two different reference genes and then the average of these two values was calculated. If a gene was identified as being expressed significantly more due to stimulation compared to the controls, the fold-change was calculated (Equation 5.4). This method for

calculation of normalisation was considered more appropriate than conventional 2-ΔΔCT method, otherwise known as the Livak method, because the Livak method does not take into account differences in reaction efficiency (Livak and Schmittgen, 2001).

$$Differentiation\ Factor = \frac{(45 - Ct\ value\ for\ reference\ gene)}{(45 - Ct\ value\ of\ reference\ gene\ for\ that\ sample)}$$

Equation 5.2 – Equation to calculate a normalisation factor.

45 = the maximum number of cycles in each qPCR run.

$$Normalised\ Expression = \frac{[(45 - Ct\ value\ for\ target\ sample) \times target\ primer\ slope]}{(Differentiation\ Factor\ for\ that\ sample\ \times reference\ primer\ slope)}$$

Equation 5.3 - Equation to calculate normalised expression.

45 = the maximum number of cycles in each qPCR run.

$$Fold\ change = 2^{(treated - control)}$$

Equation 5.4 - Equation to calculate fold-change in gene expression.

Table 5.3 – PCR primers used to discern the response of BCECs to *S.* Dublin infection or stimulation with heat-killed bacteria and LPS.

Gene symbol	Genbank ID	Primer sequence	Amplicon (bp)	Annealing temp (°C)	Final concentration in qPCR reaction (µM)	Reference
ACTB[a]	BT030480.1	F: ACGGGCAGGTCATCACCATC R: AGCACCGTGTTGGCGTAGAG	166	67	0.25	(Bougarn et al., 2011)
C2orf29[b]	XM_002691150.1	F: TCAGTGGACCAAAGCCACCTA R: CTCCACACCGGTGCTGTTCT	169	60	0.2	(Rekawiecki, Rutkowska, and Kotwica, 2012)
SUZ12[b]	NM_001205587.3	F: AGCCATGCAGGAAATGGAAG R: GCAAGAGGTTTGGCTATAGG	181	64	0.25	(Bougarn et al., 2011)
TBP[b]	NM_001075742.1	F: CAGAGAGCTCCGGGATCGT R: CCATCTTCCCAGAACTGAATAT	200	60	0.2	(Rekawiecki, Rutkowska, and Kotwica, 2012)
GAPDH[b]	NM_001034034	F: ATCTCGCTCCTGGAAGATG R: TCGGAGTGAACGGATTCG	227	60	F: 0.6 R: 0.3	(Jiménez-Pelayo et al., 2019b)
CXCL8[b]	BC103310.1	F: CCACACCTTTCCACCCCAAA R: CTTGCTTCTCAGCTCTCTTC	177	59	0.8	(Jiménez-Pelayo et al., 2019b)
TNFα[b]	AF011926.1	F: AGCCCTCTGGTTCAGACACT R: TGATGTCGGCTACAACGTG	79	60	0.25	William Roden – project student 2014
GAPDH (PCR)	NM_001034034	F: AGTTCAACGGCACAGTCAAG R: AGCAGGGATGATATTCTGGG	463	60	NA	(Blanchard et al., 2020)

Bovine caruncular epithelial cells (BCECs) were infected with *S.* Dublin or stimulated with heat-killed *S.* Dublin and *S.* Typhimurium lipopolysaccharide (LPS). The BCECs were incubated with the stimulants for 1h. RNA was harvested 2 and 24h post-stimulation. The host response was discerned using qPCR and standard PCR was used to validate primer specificity. Standard curve of 1/10, 1/100, 1/1000, 1/1,000 and 1/100,000 dilutions of cDNA (A). Standard curve of 1/3, 1/10, 1/30, 1/100, 1/300, 1/1000 dilutions of cDNA (B). Standard curve of 1/2, 1/4, 1/8, 1/10, 1/20 and 1/40 dilutions of cDNA (C).

Table 5.4 - Components of qPCR Master Mixes for each primer used, per qPCR reaction.

Target	qPCR Ready Mix (10µl)	Final conc primers (µm) and (µl added)	Water (µl)
ACTB	SyGreen	0.25 (0.5µl)	4
C2orf29	KiCqStart	0.2 (0.4µl)	4.2
GAPDH	SyGreen	F: 0.6 (1.2µl) R: 0.3 (0.6µl)	3.2
SUZ12	SyGreen	0.25 (0.5µl)	4
TBP	KiCqStart	0.2 (0.4µl)	4.2
CXCL8	KiCqStart	0.8 (1.6µl)	1.8
TNFα	KiCqStart	0.25 (0.5µl)	4

qPCR was used to discern the host response of BCECs to infection with *S.* Dublin or stimulation with either heat-killed *S.* Dublin or *S.* Typhimurium lipopolysaccharide (LPS). qPCRBIO SyGreen Mix LO-ROX (PCR Biosystems) and KiCqStart SYBR Green qPCR Ready Mix (Sigma-Aldrich).

5.2.9 - Prostaglandin E_2 Competitive Enzyme-Linked Immunosorbent Assay

Competitive prostaglandin E_2 (PGE_2) Enzyme-Linked Immunosorbent Assays (ELISAs) were performed using a competitive PGE_2 ELISA kit (ThermoFisher, catalogue number EHPGE2). The supernatants from prior infection studies were collected after 24h and frozen at -80°C as described previously and thawed prior to use in the ELISAs.

The ELISAs were performed according to the manufacturers' instructions. Briefly, standards were serially diluted 1:2 in BCEC tissue culture medium to yield a standard from 2,500pg/ml to 39.1pg/ml. Tissue culture medium was used as a blank (B_0) and in non-specific binding (NSB) wells. 100µl samples were added to a pre-coated 96-well strip plate in duplicate along with 50µl Reagent Diluent, PGE_2 Alkaline Phosphatase conjugate and PGE_2 antibody. The plate was covered with a plate sealer and incubated for 2 hours at 21°C on a plate shaker set to 500rpm. After incubation, the wells were washed three times using 400µl Wash Buffer and blotted on lint-free paper towel (Kimtech). 200µl Substrate solution was added to each well and the plate was sealed and incubated again for 45 minutes at 21°C without shaking. 50µl of Stop Solution was added to every well and

the OD was read in a Varioskan Flash spectral scanner (Thermofisher) at 405nm and 580nm.

Competitive PGE_2 ELISA results were analysed using Microsoft Excel and GraphPad Prism. ODs at 580nm were subtracted from ODs at 405nm and an average of each duplicate standard (seven in total), sample, total activity (TA), non-specific binding (NSB) and zero standard (B_0) was calculated. The NSB was then subtracted from all standards and samples, and the standard values were plotted in GraphPad Prism against the $\log_{(concentration)}$. A four-parameter logistic curve was fitted to the standards and the concentrations of PGE_2 in samples was calculated by obtaining the interpolated values from the corresponding ODs and finding their antilogs. For samples with concentrations of PGE_2 outside of the limits of detection, the inverse of the OD was plotted alongside the interpolated concentrations to discern whether samples were above or below those limits (Appendix Figure 9.13, Aii and Bii; Figure 9.14 Cii and Dii; Figure 9.15, Eii). Statistical analysis was not completed on the PGE_2 ELISA results because variation was too great.

5.2.10 - Statistical analysis

Kruskal-Wallis and Dunn's Multiple Comparisons tests were used to identify significant differences between the inocula used to infect BCECs and expression of CXCL8 and TNFα mRNA in GraphPad Prism. Fold-changes in mRNA expression were calculated once a significant difference was identified using Microsoft Excel.

5.3 - Results

The BCEC culture model was used to determine the host response to infection with four different *S.* Dublin isolates at MOIs of 1 and 10, a heat-killed inoculum of isolate 2229 at MOIs of 1 and 10 and LPS at a concentration of 1ug/ml, 2 and 24 hours post stimulation.

Samples of the inocula used during the infection studies were plated to ensure that the MOIs were as expected and to identify significant differences between isolates or between experimental replicates. Inocula did not differ significantly either between isolate or between experimental replicate in each MOI (Appendix Figure 9.10). MOIs of 1 and 10 in each isolate differed significantly from each other ($P<0.05$) apart from isolate 2229 which was approaching significance ($P=0.066$).

Conventional PCR was performed on cDNA samples from LPS-stimulated BCECs (experimental set A) using qPCR primers (Table 5.3). Sanger sequencing of these PCR products confirmed the presence of a single product, and the identities were as predicted.

5.3.1 - Assessment of suitability of reference genes

To quantify the expression of target gene mRNA, it was essential to identify suitable reference genes against which the expression of target genes can be normalised. Five candidate reference genes (ACTB, C2orf29, GAPDH, SUZ12 and TBP) were identified from the literature and all samples from one experimental set were used in qPCR reactions with these primers (Bougarn et al., 2011; Jiménez-Pelayo et al., 2019b; Rekawiecki, Rutkowska, and Kotwica, 2012) (Table 5.3).

The efficiency of TBP qPCR reactions did not fall between 90-110% and was deemed insufficient according to the MIQE guidelines for qPCR (Bustin et al., 2009). Ct values for all samples within this experimental set were obtained for ACTB, C2orf29, GAPDH and SUZ12 and assessed for their stability and suitability for normalisation.

All four candidate reference genes produced R^2 values of between 98-100% and efficiencies of between 90-110%, in line with the MIQE guidelines (Appendix Table 9.6 and Appendix Table 9.7) (Bustin et al., 2009). All four candidate reference genes also had geNorm normalisation values above 0.5 (ACTB = 1.52; C2orf29 = 1.51; GAPDH = 1.64; SUZ12 = 1.56) and were deemed unsuitable for normalisation (Vandesompele et al., 2002) (Figure 5.1 and Figure 5.2). However, due to time constraints, C2orf29 and ACTB were taken forward for use as reference genes for normalisation of target gene expression (described in Section 5.2.8.2 - Calculation of normalised target gene expression).

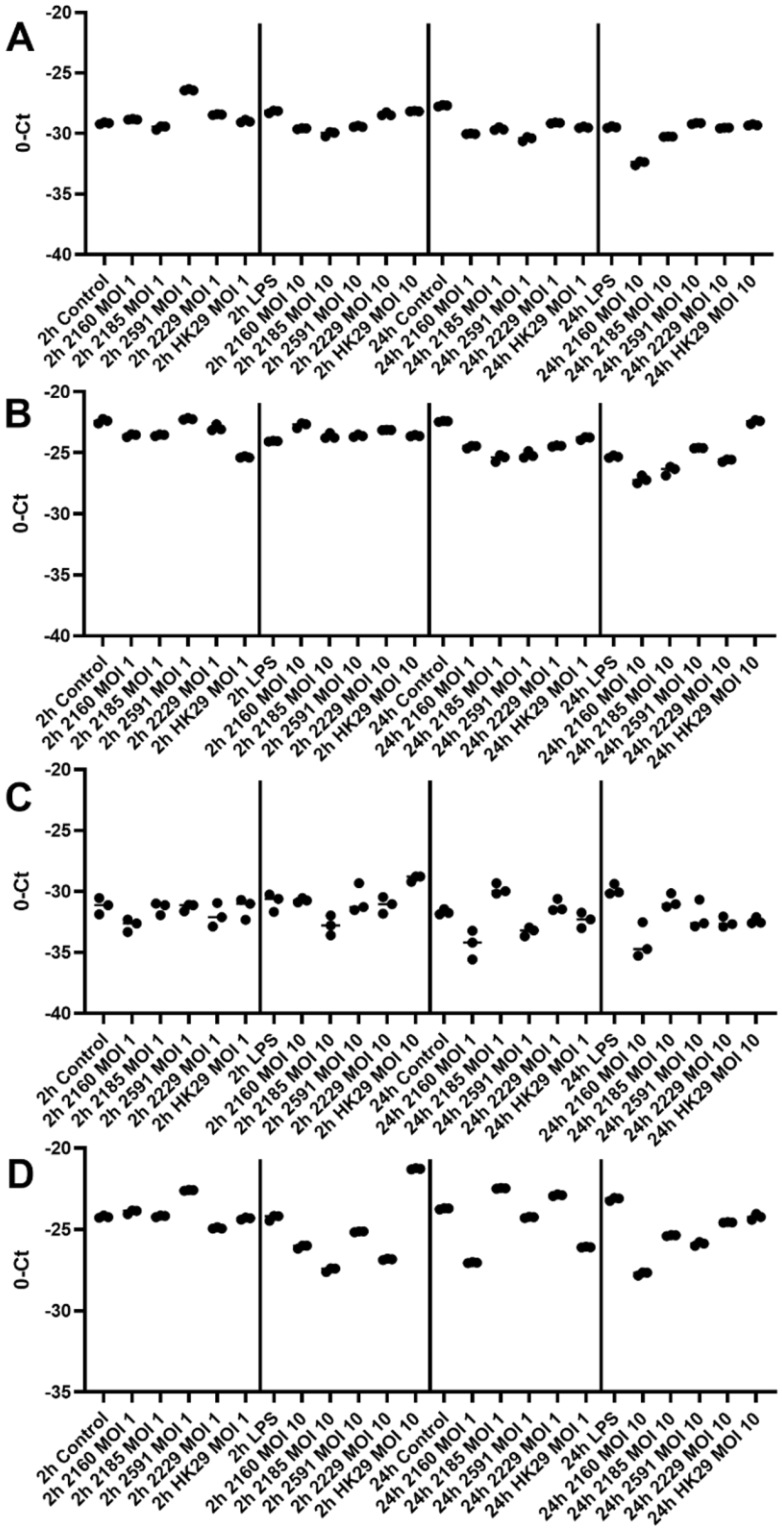


Figure 5.1 – Cycle thresholds (Ct) of candidate reference genes across all BCEC samples. Bovine caruncular epithelial cells (BCECs) were infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or *S.* Typhimurium LPS. BCECs were stimulated for 1h and RNA was collected 2 and 24h post-stimulation to quantify the host response using qPCR. Cycle Thresholds (Ct) are plotted as 0-Ct as inverting Ct improves the ease of understanding the level of expression, as a lower Ct otherwise denotes greater expression and vice-versa. C2orf29 (A), ACTB (B), SUZ12 (C) and GAPDH (D).

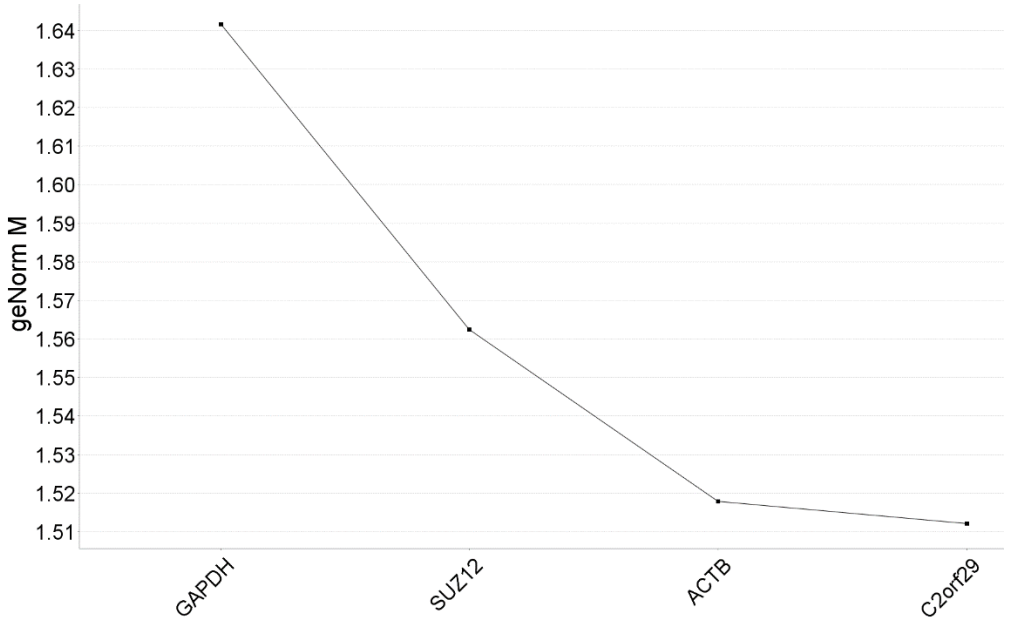


Figure 5.2 - Average expression stability of reference genes in BCECs during stimulation after 2 and 24h. Bovine caruncular epithelial cells (BCECs) were infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or *S.* Typhimurium LPS. BCECs were stimulated for 1h and RNA was collected 2 and 24h post-stimulation to quantify the host response using qPCR. Measured at 2h and 24h, with c2orf29 and ACTB being the most stable.

5.3.2 - Relative expression of CXCL8 mRNA increases during *S.* Dublin infection

The chemokine CXCL8 is secreted by a variety of cell types including epithelial cells and is associated with pro-inflammatory responses to infection (Eckmann, Kagnoff, and Fierer, 1993). The expression of CXCL8 mRNA was investigated in BCECs stimulated with heat-killed and live *S.* Dublin isolates and LPS from *S.* Typhimurium as an indicator of a pro-inflammatory response to infection.

Standard curves were obtained from all five experimental sets for CXCL8, with R^2 and efficiency values falling within the limitations set out by the MIQE guidelines (Bustin et al., 2009) (Appendix Table 9.8).

CXCL8 mRNA was detected in all samples irrespective of stimulation at both 2h and 24h post stimulation (Figure 5.3). After 2h of stimulation, expression of CXCL8 mRNA was the same across all stimulations (Figure 5.3). After 24h, all live-infected BCECs produced elevated average relative expression of CXCL8 mRNA compared to

the controls but only four reached statistical significance (Figure 5.3). Infection with L 2160/17 at an MOI of 1 resulted in 26-fold higher expression of CXCL8 mRNA (P=0.0414) and BCECs infected with isolates L 2185/17, L 2591/17 and 2229 at an MOI of 10 expressed 77-fold (P=0.0040), 51-fold (P=0.0243) and 38-fold (P=0.0123) more CXCL8 mRNA compared to controls (Figure 5.3). Infection with isolate L 2160/17 at an MOI of 10 increased CXCL8 mRNA expression by 28-fold but did not meet the significance threshold (P=0.0687) (Figure 5.3). Expression of CXCL8 mRNA was relatively consistent across the different isolates and in spite of different MOIs (Figure 5.3). BCECs expressed similar levels of CXCL8 mRNA after 2h of stimulation with LPS and heat-killed isolate 2229 at MOIs of 1 and 10 (Figure 5.3). The experimental procedure included media changes at 2h, removing LPS and heat-killed isolate 2229 at this point during the 24h infection experiment. After 24h, there was no significant difference in CXCL8 mRNA expression in BCECs in response to LPS and heat-killed isolate 2229 at an MOI of 1 or 10 (Figure 5.3).

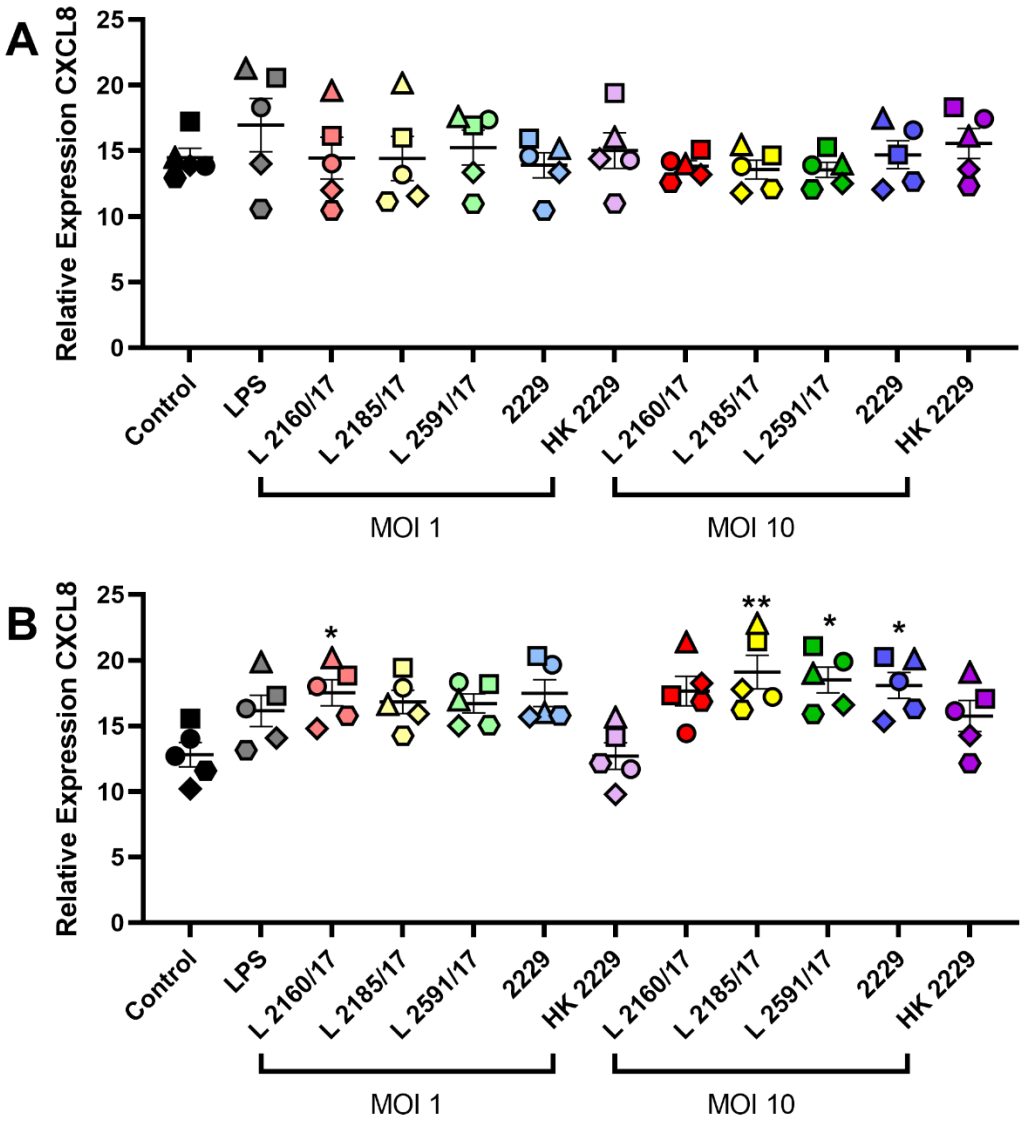


Figure 5.3 – Relative expression of CXCL8 mRNA in BCECs in response infection with *S.* Dublin or stimulation with heat-killed *S.* Dublin or *S.* Typhimurium lipopolysaccharide (LPS) at 2h (A) and 24h (B) post stimulation. Bovine caruncular epithelial cells (BCECs) were stimulated for 1h and RNA was collected at 2 and 24h post-stimulation to discern the host response to stimulation. Asterisks denote statistically significant difference compared to control according to Kruskal-Wallis and Dunn's multiple comparisons tests (* = P<0.05, ** = P<0.005). Individual experimental sets are denoted by different shapes (circle = A (passage 40), square = B (passage 40), triangle = C (passage 32), diamond = D (passage 32), hexagon = E (passage 35)). "HK" = heat killed. MOI = multiplicity of infection. Reference genes used for normalisation were ACTB and C2orf29. n=5.

5.3.3 - Expression of TNFα mRNA increases during infection with *S.* Dublin

Like CXCL8, TNFα expression is associated with pro-inflammatory immune responses which have been implicated in reproductive failure like abortion events. Therefore, the expression of TNFα in response to infection with *S.* Dublin was investigated as a marker of a potential pathway from infection to abortion.

Standard curves were obtained from five experimental sets for TNFα and R^2 and slope values were within the limitations set out by the MIQE guidelines for the four sets included in analysis (Bustin et al., 2009) (Appendix Table 9.9).

TNFα mRNA was detected in most samples, including unstimulated controls 2 and 24h post stimulation, and expression was highly variable between the four experiments (Figure 5.4). At 2h, six of the eight *S.* Dublin live-infected samples in experimental set E did not contain any TNFα mRNA and all other samples in this set have lower relative expression rates compared to the other sample sets, including the control (Figure 5.4). After 24h, clear increases in average relative expression of TNFα mRNA were observed in all stimulations apart from heat-killed isolate 2229 at an MOI of 1 (Figure 5.4). TNFα mRNA was significantly upregulated in samples infected with isolates L 2185/17, L 2591/17 and 2229 at an MOI of 10, with 3133-fold (P=0.0147), 2532-fold (P=0.0438) and 2066-fold (P=0.0291) respectively more mRNA detected compared to controls (Figure 5.4). Infection with isolate L 2160/17 at an MOI of 10 increased TNFα mRNA expression 1259-fold and was nearing statistical significance (P=0.0816) (Figure 5.4). The expression of TNFα mRNA in live-infected BCECs was similar across the different isolates and MOIs (Figure 5.4). As previously described in CXCL8, the experimental procedure for the 24h time course included conducting media changes at 2h which removed media containing *S.* Typhimurium LPS and heat-killed isolate 2229. Despite this, clear increases in TNFα mRNA were observed in BCECs stimulated with LPS and heat-killed isolate 2229

at an MOI of 10 (Figure 5.4). Stimulation with heat-killed isolate 2229 at an MOI of 1 yielded TNFα mRNA similar to the control (Figure 5.4).

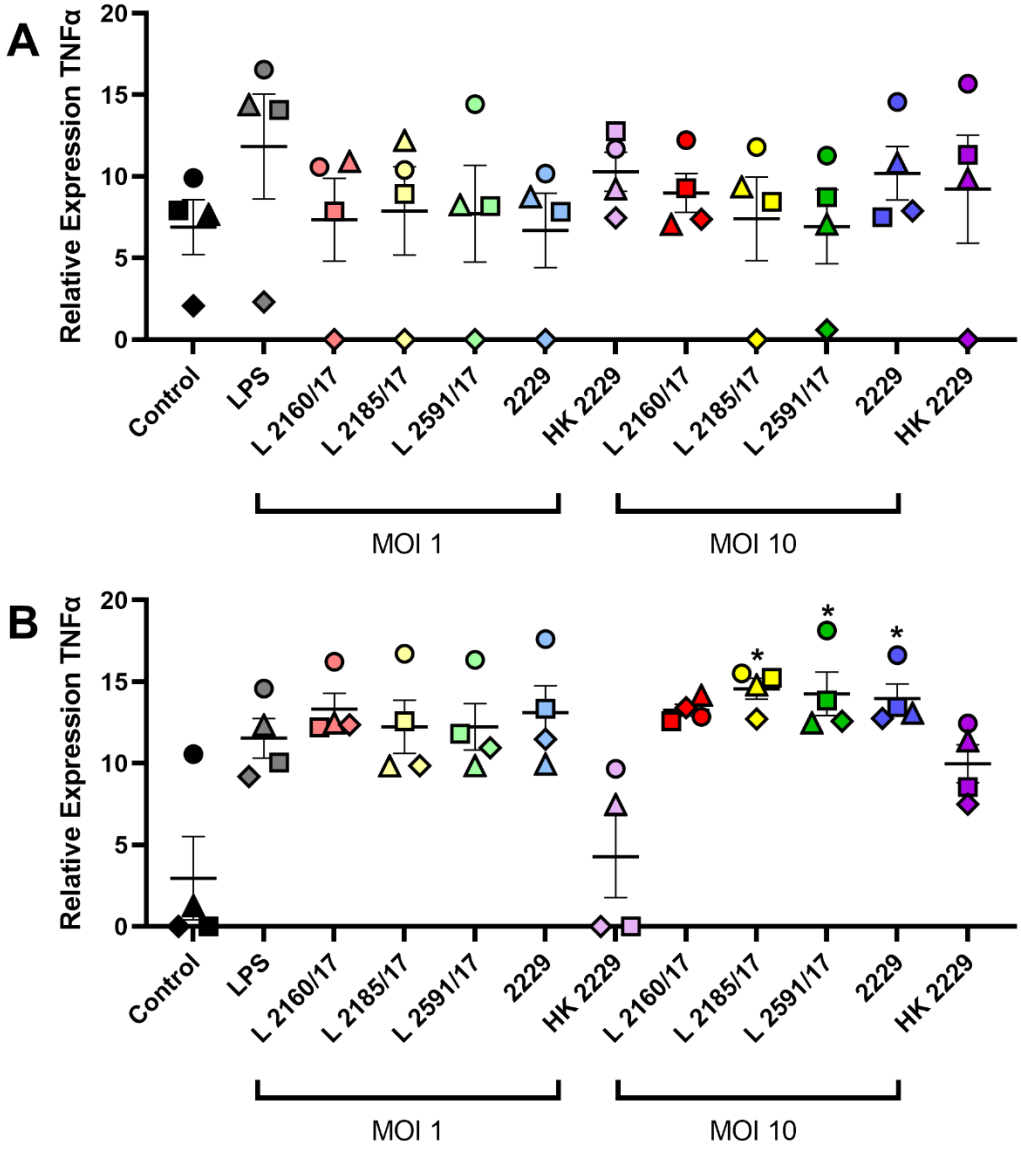


Figure 5.4 – Relative expression of TNFα mRNA in BCECs in response to infection with *S.* Dublin or stimulation with heat-killed *S.* Dublin or *S.* Typhimurium lipopolysaccharide (LPS) at 2h (A) and 24h (B) post stimulation. Asterisks denote statistically significant difference compared to control according to Kruskal-Wallis and Dunn's multiple comparisons tests (* = P<0.05). Individual experimental sets are denoted by different shapes (circle = A (passage 40), square = B (passage 40), triangle = C (passage 32), diamond = E (passage 35)). "HK" = heat killed. MOI = multiplicity of infection. Reference genes used for normalisation were ACTB and C2orf29. n=4

5.3.4 - BCECs produce PGE_2 in response to bacterial stimulation after 24h

PGE_2 is important during both the immune response as a pro-inflammatory cytokine which biases the response towards a Th2 type response, and as a component of the complex hormonal balance that exists during pregnancy. Increases in PGE_2 have been associated with early labour, whilst its downstream impact of increasing protective IL-10 transcription in macrophages.

The concentration of PGE_2 produced in response to stimulation with live and heat-killed *S.* Dublin isolates and LPS from *S.* Typhimurium were assayed by competitive ELISA 24h after initial stimulation.

Seven-point standard curves were generated for all assays with concentrations of PGE_2 (Appendix Figure 9.12). In the third standard curve, an outlier value for the highest standard (2,500pg/ml) was identified by comparing this curve to the other two generated and the manufacturers instructions. This value was replaced during data analysis with the average value of the corresponding standards from the other standard curves (Appendix Figure 9.12). The non-linear fit (R^2) values for these curves were between 0.9975 and 0.9998.

A trial was conducted using neat, 1:2 and 1:4 dilutions of control and LPS-stimulated supernatants from experimental set A to discern whether samples required dilution. LPS-stimulated cells should theoretically produce a strong immune response as LPS is a primary PAMP to which epithelial cells can respond (Silva et al., 2012). The results from this trial suggested that dilution of the samples was not necessary, corroborating the manufacturers instructions (Figure 5.5, A – "Cont" and "LPS", both undiluted results).

After 24h, PGE_2 was detected in all but five supernatant samples (two control samples, two heat-killed *S.* Dublin isolate 2229 at an MOI of 1 and one heat-killed *S.* Dublin isolate 2229 at an MOI of 10) across the five experimental sets (Figure 5.5). BCECs produced PGE_2 in response to infection with live *S.* Dublin isolates and 17 out of 40 live-

infected BCEC samples produced more than 4,000pg/ml PGE_2, above the reliable limit of quantification in this assay (Figure 5.5, denoted by hashed bars). Whilst it is likely that PGE_2 is produced in response to *S.* Dublin, the variation in concentrations between different experimental sets prevented statistical analysis (Appendix Figure 9.13, Ai and Bi; Figure 9.14 Ci and Di; Figure 9.15, Ei). For example, the concentration of PGE_2 produced in response to isolate L 2160/17 MOI 1 varied between experiments from 448.66pg/ml to <4,000pg/ml (Figure 5.5). PGE_2 production was also highly variable between stimulations in different experiments. For example, in experimental set A, infection with isolates L 2185/17 and L 2591/17 (MOI 1) yielded 407.25pg/ml and 3,513.71pg/ml PGE_2 respectively, a difference of nine-fold (Figure 5.5, A). However, in experimental set E, infection with the same isolates yielded 1579.77pg.ml and 1605.79pg/ml PGE_2 respectively, concentrations much more similar compared to experimental set A (Figure 5.5, E).

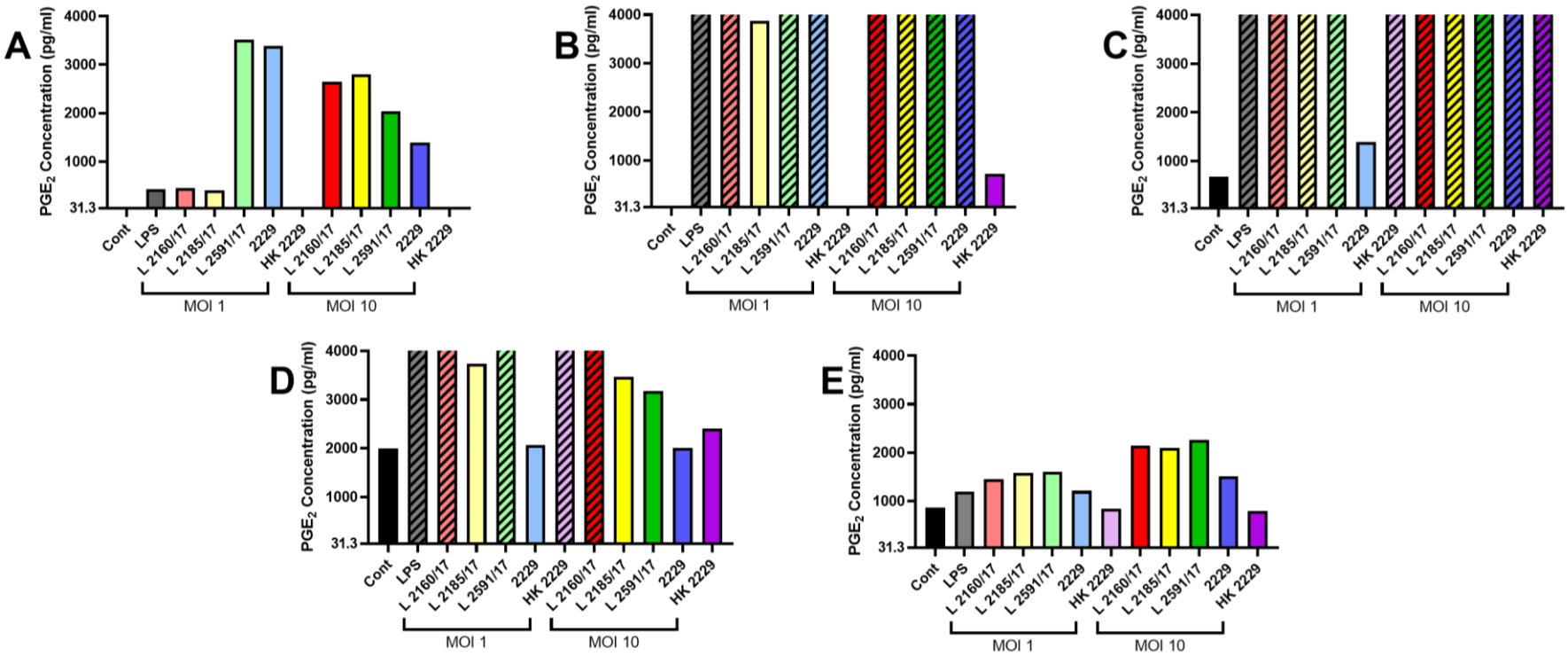


Figure 5.5 – Concentrations of PGE_2 produced by BCECs infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or LPS after 24h, measured by competitive ELISA. Five infection/stimulation experiments (A-E) were conducted using Bovine Caruncular Epithelial Cells (BCECs) and *S.* Dublin isolates at multiplicities of infection (MOIs) of 1 and 10. After 24h of infection/stimulation, the supernatants were removed and stored at -80°C until use. Competitive Enzyme linked immunosorbent assays (ELISAs) were conducted to quantify the concentration of PGE_2 produced by the BCECs in response to the bacteria or stimulation regimen. Hashed bar denotes the concentration exceeded 4,000pg/ml, the reliable limit of quantification in the ELISA. Cells used in experimental sets A and B were passage 40, C and D were passage 32 and E were passage 35.

5.4 - Discussion

The precise molecular pathways which result in abortion due to infection with *S.* Dublin in cattle are yet to be identified. Investigation of CXCL8, TNFα and PGE_2 aids in understanding the mechanisms underpinning an abortion and could contribute to preventing these events in the future.

CXCL8 was upregulated by BCECs in response to infection with different *S.* Dublin isolates 24h post infection. CXCL8 is a key component of the proinflammatory response to infection and is produced by a variety of tissues, including epithelial cells (Eckmann, Kagnoff, and Fierer, 1993). CXCL8 is a potent chemotactic agent and activator for neutrophils, and is involved in essential processes during pregnancy, including cervical ripening at parturition and development of the corpus luteum (Talbott et al., 2014; Van Engelen et al., 2009). CXCL8 is reported to increase in the placentomes as pregnancy progresses but significant increases in CXCL8 expression due to infection are often associated with poor pregnancy outcomes (Van Engelen et al., 2009). For example, increased CXCL8 expression is associated with severe placental histopathology in *Brucella abortus* infected cattle (Carvalho Neta et al., 2008). Challenge with abortifacient *Escherichia coli* and *Truperella pyogenes* results in increased CXCL8 secretion in bovine endometrial tissues (Borges, Healey, and Sheldon, 2012). *Chlamydia abortus* infection of ovine trophoblast cells results in an increase in CXCL8 production (Wheelhouse et al., 2009). BCECs upregulate CXCL8 mRNA in response to *Neospora caninum,* and during challenge with heat-killed *S.* Dublin, fragments of intercotyledonary and endometrial tissues upregulate CXCL8 mRNA expression (Jiménez-Pelayo et al., 2019b; Silva et al., 2012). Increased CXCL8 in many of these pathologies is associated with infiltration of neutrophils in the placenta, including infection with *Chlamydia abortus* in sheep and *Neospora caninum* in cattle (Navarro et al., 2004; Regidor-Cerrillo et al., 2014; Sammin et al., 2006). Placental infiltration by neutrophils is also observed in infection with *Bacillus licheniformis* but this is yet to be associated

with increased CXCL8 expression (Agerholm et al., 1999). Large numbers of infiltrating neutrophils have been reported as part of the pathogenesis of infection with *S.* Dublin in various tissues, including in the placentomes of experimentally infected pregnant cattle (Hall and Jones, 1977; Pecoraro, Thompson, and Duhamel, 2017). Whilst neutrophils are the first immune cells present at the site of infection and are essential for the clearance of *Salmonella,* it is possible that these cells could also damage the placental structures (Entrican, 2002; Richter-Dahlfors, Buchan, and Finlay, 1997; Vazquez-Torres et al., 2004). Neutrophils can release proteolytic enzymes and reactive oxygen species into the extracellular matrix, causing severe tissue damage and an up-regulation in the local inflammatory response as a result (Iba et al., 2013; Rydell-Törmänen, Uller, and Erjefält, 2006). Damage to a proportion of the placentomes because of an inappropriate neutrophil response could damage the delicate feto-maternal interface, leading to insufficient nutrient and gas exchange and subsequently abortion (Longbottom and Coulter, 2003).

Increased expression of proinflammatory TNFα mRNA was also observed in BCECs infected with different isolates of *S.* Dublin after 24h post infection. This is unsurprising as infections with *Salmonella* are often associated with upregulation of TNFα expression as LPS is a TLR4 ligand which, when activated, initiates NF-κβ signalling leading to the production of TNFα (Tili et al., 2007). BCECs have been shown to upregulate expression of TNFα in response to *Neospora caninum* infection, demonstrating this tissues ability to initiate pro-inflammatory responses to two reproductive infections (Jiménez-Pelayo et al., 2019b). TNFα is essential for pathogen clearance during infections with *Listeria monocytogenes* and *Chlamydia pneumoniae* (Barber, Fazzari, and Pollard, 2005; Laster, Wood, and Gooding, 1988; Njau et al., 2009). In pregnant sheep, infection with *Chlamydia abortus* is thought to be controlled by the presence of TNFα, where its absence could allow for infection of the fetus (Entrican, Buxton, and Longbottom, 2001). However, the TNFα produced in response to LPS in chlamydial infections of trophoblast cells are associated with

production of PGE_2 which in turn may aid in inducing fetal expulsion (Kerr et al., 2005; Longbottom and Coulter, 2003). In cattle, placental TNFα mRNA is upregulated during *Neospora caninum* infections and higher numbers of TNFα expressing immune cells are observed in the maternal caruncular tissues and blood vessels of dams carrying deceased offspring (Cantón et al., 2014a; Rosbottom et al., 2008). In sheep, *Salmonella enterica* serovars Abortusovis and Dublin are associated with ovine abortion and both cause significant up-regulation of TNFα mRNA in the spleen and draining lymph nodes when injected subcutaneously (Montagne et al., 2001). Generally, TNFα is considered to be deleterious to pregnancy and up-regulation of TNFα expression is often seen alongside abortion (Dealtry, O-Farrell, and Fernandez, 2000). Recruitment of immune cells and initiation of necrosis or apoptosis in the feto-maternal interface, as with CXCL8, could cause tissue damage and therefore restrict gas and nutrient exchange in the placentomes, leading to fetal death and expulsion (Longbottom and Coulter, 2003). However, as the production of TNFα is imperative for pathogen clearance, it is not entirely clear to what extent the production of TNFα here could initiate an abortion event. Further work with a more complex model of the reproductive tract is required to discern the extent to which TNFα is involved with causing an abortion.

Whilst BCECs here (similar to bovine endometrial epithelial cells) appear to constitutively express CXCL8 and TNFα, there are clear increases in expression observed when infected with *S.* Dublin (Chanrot et al., 2017). It is possible that the increase in CXCL8 and TNFα above usual, homeostatic or pregnancy-promoting levels could lead to downstream immune activation which could result in the pathologies described in an abortion event (Hall and Jones, 1976). Downstream immune activation could include apoptosis and restricted placental blood flow as in chlamydial abortions in sheep and *S.* Enteritidis infection in pregnant mice (Betancourt et al., 2021; Entrican, 2002; Llana et al., 2014).

The production of PGE_2 by BCECs in response to infection with *S.* Dublin could not be statistically assessed in these experiments because of high variability across the different experimental repeats. However, BCECs produced PGE_2 in most scenarios involving the different stimulants after 24h which was to be expected. PGE_2 is generally thought as a regulator of the immune system which prevents unnecessary and damaging inflammation, although this is not always the case (Agard, Asakrah, and Morici, 2013; Martínez-Colón and Moore, 2018). Immunosuppressive and regulatory actions tend to be correlated to the action of PGE_2 on EP2 and EP4 receptors, two of the four specific EP receptors for which PGE_2 is a ligand (Fujino, Salvi, and Regan, 2005). Evidence of the impact of PGE_2 on infection of the pregnant bovine reproductive tractor reproductive tissues is limited, though studies have been conducted into various targets of PGE_2 which are relevant. PGE_2 has been identified along with progesterone and oestradiol 17b as being part of a hormonal imbalance which occurs due to Chlamydial infection in sheep (Leaver et al., 1989). Increases of PGE_2 and oestradiol in association with decreasing progesterone is thought to initiate parturition earlier and lead to the abortions and stillbirths in late gestation characteristic of this infection (Leaver et al., 1989). It is possible that damage to the placentomes, organs which produce all three of these hormones during pregnancy, as a result of infection leads to fetal death and abortion because of this hormonal imbalance during *S.* Dublin infection.

As previously described, neutrophils are essential for the clearance of *Salmonellae* and are recruited to the placentomes during *S.* Dublin infection in the bovine pregnant uterus (Hall and Jones, 1976; Richter-Dahlfors, Buchan, and Finlay, 1997). However, during infection with *Listeria monocytogenes,* murine neutrophils pre-treated with PGE_2 were less able to kill bacteria via decreased cellular migration, reduced reactive oxygen species production and reduced bacterial uptake compared to the control (Pitts and D'Orazio, 2019). Furthermore, PGE_2 influences the progression of NETosis, a process

distinct from apoptosis or necrosis during which neutrophils release neutrophil extracellular traps (NETs) to capture and kill bacteria (Brinkmann et al., 2004). Treatment of PGE_2 inhibits human neutrophils from producing NETs via stimulation of EP2 and EP4 receptors, hindering their bactericidal activity (Shishikura et al., 2016). Careful regulation of NETosis must occur as a protective mechanism for the host, as NETosis can lead to epithelial cell damage and is associated with a range of different inflammatory diseases in humans (Cahilog et al., 2020; Saffarzadeh et al., 2012). NETosis may already be hindered by the presence of high concentrations of progesterone during pregnancy (Giaglis et al., 2016). However, without this mechanism of bacterial clearance, an infection could progress unencumbered which would ultimately lead to tissue damage too.

More generally, PGE_2 suppresses the production of IL-12, thereby reducing the activity of natural killer cells and decreasing the downstream production of IFNγ, both of which are important in the clearance of intracellular pathogens like *Salmonellae* (Betz and Fox, 1991; Hilkens et al., 1995; van der Pouw Kraan et al., 1995; Walker and Rotondo, 2004). This reduction in IFNγ is also implicated in suppression of NK cell activation of macrophages which too are impacted by PGE_2 through a reduction in bactericidal activity via decreased radical production (Asakrah et al., 2013; Mailliard et al., 2005; Serezani et al., 2012). During pregnancy, a Th2 biased state of immunity is maintained in order to protect the allogenic fetus from the maternal immune system, whilst maintaining maternal immunocompetence to an extent (Kaliński et al., 1997; Oliveira et al., 2013). However, the immunosuppressive impact of this Th2 bias coupled with increasing immunoregulatory PGE_2 in response to *Salmonella* infection could reduce the ability of the mother to clear the invading pathogen by preventing essential pro-inflammatory processes (Bowman and Bost, 2009). Specifically, PGE_2 acting on prostaglandin receptors 2 and 4 can increase immunosuppressive IL-10 which is associated with bacterial colonisation, along with

decreasing TNFα expression (Akaogi et al., 2004; Montagne et al., 2001; Shinomiya et al., 2001). Whilst the experiments presented here cannot contribute to the knowledge base in the literature, it is likely that further investigation into the host-response to *S.* Dublin would demonstrate the production of PGE_2 in response to infection.

The production of CXCL8 and TNFα by BCECs was similar across the different *S.* Dublin isolates after 24h of infection. This was unexpected as the isolates were selected because of their differences between one another but is perhaps unsurprising because all of the isolates were previously associated with bovine infection and are therefore capable of infection. More surprising was that the cytokines measured were expressed at similar levels even when challenged with ten fold more bacteria, whilst a dose-dependent increase in PGE_2 was observed with BCECs challenged with *Listeria monocytogenes* and *Leptospira borgpetersenii* (Collet et al., n.d.). Extracellular bacteria were killed after 1h so it is possible that the higher dose of *S.* Dublin may have elicited a greater immune response if allowed to interact with the BCECs for longer. Equally, as described previously, it appears as though bacteria are limited in their growth over the course of 24h intracellularly (chapter reference). Longer stimulation studies without the same wash steps as conducted here may be required to identify the impact of LPS and heat-killed *S.* Dublin isolates at MOIs of 1 and 10, as the stimulants were removed after 1h in line with the protocols of the live infected cells.

Whilst changes in mRNA expression give a good indication of the transcriptional environment, this does not necessarily directly correlate to increases in active protein in response to infection. For example, post-transcriptional repressor miRNA miR-125b targets TNFα mRNA to prevent host damage caused by inappropriate inflammatory responses (Tili et al., 2007). Even when measuring active protein such as PGE_2 by competitive ELISA, protein turnover (in this case mediated by 15-hydroxyprostaglandin dehydrogenase) and clearance may be more rapid *in vivo* compared to this model, so

a proportion of the increase observed may be due to a lack of degradation (Kalinski, 2012). Similarly, it is not possible in this model to determine whether the concentrations of CXCL8, TNFα and PGE_2 are biologically relevant, in part because these cytokines must act on specific receptors which must also be present to elicit their responses on different cell types. Logically speaking, an increase in proinflammatory cytokines CXCL8 and TNFα and immunoregulatory hormone PGE_2 in response to infection with *S.* Dublin are to be expected – infections with *Salmonellae* in a variety of species with various serovars tend to result in an inflammatory response, and these infections are associated with a detrimental outcome. Therefore, the CXCL8 and TNFα results can act as a foundation upon which further research can be conducted into the precise mechanisms underpinning an abortion due to *S.* Dublin.

Of the four candidate reference genes tested for qPCR, none reached the threshold for normalisation which ultimately introduces limitations of the qPCR data. Due to time constraints, the recommended panel of ten reference genes could not be evaluated, so the five genes evaluated were identified from the literature. Most pressing was identification of suitable reference genes from cattle, preferably those identified in reproductive tissues. C2orf29 and TBP were both identified and validated using GeNorm as the most stable candidate reference genes in the bovine corpus luteum (Rekawiecki, Rutkowska, and Kotwica, 2012). Similarly, SUZ12 and ACTB were identified as stable reference genes in bovine mammary epithelial cells, including during infection with *Escherichia coli* and *Staphylococcus aureus* (Bougarn et al., 2011). Finally, GAPDH and ACTB were used as reference genes in BCECs infected with *Neospora caninum*, not only using the same cells as proposed in this study but additionally in an infection context (Jiménez-Pelayo et al., 2019b).

Aside from the possibility that these genes may be impacted by the infection process, these genes may not have met a stability threshold because of issues in processing the RNA samples and cDNA synthesis.

The methods used here considered the presence of contaminating DNA and potential differences in RNA yield from the extraction process. However, due to time constraints, other RNA quality parameters were not accounted for which could have an impact on downstream gene quantification. These RNA quality parameters include contaminating protein (using the A260/A280 method, (Glasel, 1995), salts and other organic contaminants (using the A260/A230 method, (Warburg and Christian, 1942) and assessing RNA integrity (using gel electrophoresis and looking for clear 18S and 28S bands, (Sambrook, Fritsch, and Maniatis, 1989). Furthermore, the concentration of synthesised cDNA was assumed to be 1µg/ml as the volume of RNA added for these reactions was adjusted based on yields measured by Qubit (Thermofisher). This assumed the enzymatic reactions of all samples were identical and would produce a ratio of RNA:cDNA of 1:1 and that the Qubit was accurate for every sample.

The variation in the expression of PGE_2 measured by ELISA prevented statistical analysis of the response of BCECS to infection or stimulation with *S.* Dublin. Whilst dilution could have been beneficial for some samples, this would have been inappropriate for others and may have prevented quantification at lower concentrations. It would not have been possible to predict this variation and therefore which samples required dilution without some sort of quantification first which would also not have been possible as freeze-thawing the samples is not recommended. For future experiments, supernatants should be frozen in smaller aliquots or subsets to allow for initial and final optimised quantification of PGE_2. There was also variation in TNFα and CXCL8 expression across the different experimental sets despite being conducted under identical experimental protocols. For example, BCECs in experimental set E expressed consistently lower amounts of CXCL8, TNFα and PGE_2 compared to the other experimental sets. These cells were passage 35, in between sets A and B (passage 40) and sets C and D (passage 32) and were in culture for a similar number of days as set D (18 and 16 days respectively) compared to other experimental sets. However, the cells used in set E were frozen

for storage and revived only five days later, a very short period of time compared to other cell sets, some of which were frozen for 8 years before being thawed for this work. This difference in cryopreservation length and potential differences between methodology of cryopreservation conducted by different people prior to the use of these cells could introduce variation in cell viability and responses (Shaik et al., 2018).

Future work investigating the host response to infection with *S.* Dublin would ideally be conducted in a more complex model, or in whole animal studies. Use of an *ex vivo* organ culture (EVOC) of the bovine placentome during an infection with *S.* Dublin would allow for greater characterisation of the host response in multiple cell types. This is particularly relevant as maternal and fetal tissue has been reported to respond to immune challenges differently, yet could influence one another (Jiménez-Pelayo et al., 2019b). Using transcriptomics could aid in understanding the complex interplay of immune factors in the placentome during infection without needing to predict those changes beforehand, as was necessary here using qPCR. EVOC transcriptomics could then be associated with histological investigation of the impact of the bacteria in the placentome. The EVOC would not account for the impact of the wider host response, including infiltrating neutrophils and macrophages which are likely to have a large impact on the pathology associated with *S.* Dublin infection (Hall and Jones, 1977). Therefore, animal studies and histological investigation of infiltrating immune cells into the placentome could be hugely beneficial. Furthermore, there is little evidence underpinning the pathological changes that occur in the bovine placentome during infection with and subsequent abortion due to *S.* Dublin. Whole animal studies could also allow for investigation into the Th2 biases during pregnancy in the placentome by looking for infiltrating cells expressing high levels of immunoregulatory cytokines like IL-10 rather than proinflammatory cytokines. Further investigation into the impact of infiltrating neutrophils could also be important as these cells can cause damage to host tissues. The increase in CXCL8 reported in

this study and evidence of significant neutrophil infiltration reported in placental pathology during infection warrants further investigation, including the impact of PGE_2 on these cells. Understanding how PGE_2 impacts the efficiency of neutrophil killing *S.* Dublin would provide an insight into the immunoregulatory mechanisms which may protect the mother but equally could allow for unmarked bacterial colonisation.

5.5 - Summary

BCECs upregulate pro-inflammatory (CXCL8 and TNFα) cytokines in response to infection with *S.* Dublin to facilitate pathogen clearance whilst preventing host tissue damage and maintaining pregnancy. However, the subsequent impact of this immune response appears to either be insufficient whereby the bacteria are unencumbered by this response and can colonise the host tissues, killing the fetus in the process, or the host responds inappropriately and initiates the abortion event to protect the dam. Further work should aim to characterise the host response in a more complex model of the pregnant bovine reproductive tract and evaluate the impact of neutrophils on host tissue integrity. Furthermore, improved investigation into host-produced regulatory cytokines like PGE_2 would improve understanding of how abortion events occur.

Chapter 6 - Identifying antimicrobial resistance in *Salmonella* Dublin and *Salmonella* Typhimurium

6.1 - Introduction

Antimicrobial resistance (AMR) is a global issue affecting agriculture and medicine, human and animal health (World Health Organisation, 2015). It has been estimated that 700,000 people die from previously treatable diseases every year because of AMR, and this is predicted to increase without serious changes to the way we use antimicrobial substances (O'Neill, 2014, 2016). The World Health Organisation have recommended that particular classes of antibiotics should not be used in animal medicine at all, and others should only be used as a last resort in animal medicine because of their importance in human medicine (World Health Organisation, 2017). The Responsible Use of Medicines in Agriculture Alliance (RUMA) recommend avoiding the use of antibiotics in favour of improving biosecurity and animal management practices (Responsible Use of Medicines in Agriculture Alliance, 2015). However, it is sometimes necessary for an animal to be treated with antibiotics alongside fluid therapy to improve the health status of the animal more quickly and reduce the impact on the individuals welfare.

Some of the most commonly purchased classes of antibiotics in cattle in the UK include beta-lactams, aminoglycosides, amphenicols, tetracyclines, trimethoprim/sulphonamides and fluoroquinolones (Veterinary Medicines Directorate, 2016a). In England and Wales, *Salmonellae* isolated from cattle are most commonly resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide compounds and tetracyclines (Veterinary Medicines Directorate, 2016b).

Resistance to ampicillin, chloramphenicol, nalidixic acid, neomycin, streptomycin, sulphonamide compounds and tetracycline in *S.* Dublin isolates from all food producing animals in England and Wales is observed occasionally and may be increasing over time (Veterinary Medicines Directorate, 2016b). The prevalence of antimicrobial resistance in *S.* Typhimurium isolates from livestock in the UK is comparatively high. More isolates are found to be resistant to the same antibiotics as *S.* Dublin isolates, as well as apramycin, cefotaxime, ceftazidime, ciprofloxacin and gentamycin (Veterinary Medicines Directorate, 2020).

Currently, antimicrobial resistance is monitored using susceptibility testing in a proportion of the *S.* Dublin isolates identified in clinical presentations in cattle. Therefore, it is difficult to understand the potential AMR reservoir as the panel of antibiotics is limited and no data or susceptibility testing is routinely conducted outside of this panel. Furthermore, very little work has been done in *S.* Dublin antimicrobial resistance, whilst *S.* Typhimurium is often used as a model organism in AMR research.

6.1.1 - Aims and hypotheses

This chapter aims to characterise the antibiotic sensitivity of 16 *S.* Dublin isolates from the UK to antibiotics commonly used in farming. AMR genes will be identified in these 16 isolates and compared to a larger set of 250 *S.* Dublin isolates to understand if these 16 isolates are representative. Furthermore, the resistances identified in *S.* Dublin will be compared to those in *S.* Typhimurium as a host generalist and more widely studied serovar, with similar numbers of isolates from different origins of isolation to identify discrepancies.

We hypothesise that there will be little, if any phenotypic antimicrobial resistance observed in *S.* Dublin isolates, in keeping with current UK trends. It is possible that AMR genes will be identified in the *S.* Dublin isolates, but the number and prevalence of AMR genes will be greater in *S.* Typhimurium isolates.

6.2 - Materials and Methods

16 *S.* Dublin isolates characterised previously in Chapter 3 were used for phenotypic antibiotic sensitivity testing. These isolates were genotypically analysed along with the other 250 *S.* Dublin isolates and 266 *S.* Typhimurium isolates for the presence of AMR genes.

6.2.1 - Antibiotic Sensitivity Testing

Antibiotic sensitivity testing was conducted by 3rd year Veterinary Medicine student Stijn Brussen under the supervision of Jemma Franklin whilst in the laboratory. Bacterial cultures were grown overnight in a shaking incubator (Thermofisher Forma Orbital Shaking Incubator) at 37°C, and 100µl of each sample was spread evenly over Mueller Hinton (Sigma Aldrich, UK) agar plates. Antibiotics were chosen based on their frequent purchase and use in cattle and their potential resistance according to surveillance reports (Veterinary Medicines Directorate, 2016a). Antibiotic discs were placed onto the agar and the cultures were incubated for 16 hours at 37°C (Table 6.1). The diameter of the zone of inhibition was then measured and recorded. All experiments were performed in triplicate. Anomalous results such as uneven bacterial growth around the antibiotic disc were repeated (Figure 6.1).

Table 6.1 - Antibiotics used to test sensitivity of *S.* Dublin in disc diffusion assays.

Antibiotic	Class	Importance to human health (WHO)	Concentration (µg)
Tetracycline	Tetracyclines	Highly important	30
Streptomycin	Aminoglycosides	Critically important	10
Chloramphenicol	Phenicols	Highly important	30
Amoxicillin-clavulanic acid	B-lactams	Critically important	30
Trimethoprim-sulfamethoxazole	Sulfonamides	Highly important	25
Nalidixic acid	Quinolones	Critically important	30

Antibiotics were selected based on their likelihood of use in treatment of Salmonellosis or other enteric diseases in cattle. Human importance as denoted by the World Health Organisations 2018 report on antibiotic usage.

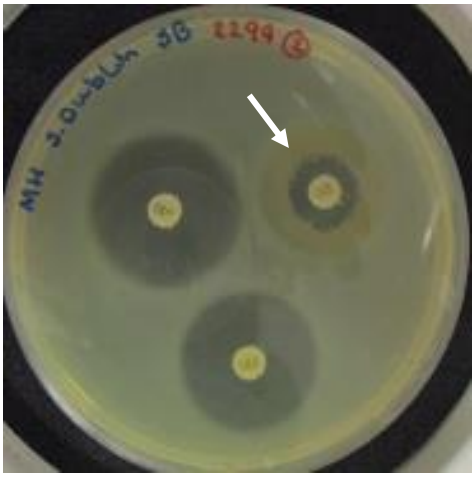


Figure 6.1 – Growth of *S.* Dublin isolate 2229 on Mueller Hinton agar plates with antibiotic discs. White arrow indicates an area of abnormal bacterial growth around a Nalidixic Acid antibiotic disc, with single colonies growing in the zone of inhibition and abnormal colouration of colonies surrounding the disc.

6.2.2 - Identification of Antimicrobial Resistance Genes

Data cleansing and AMR gene categorisation for *S.* Dublin isolates was completed by 3rd year Veterinary Medicine student Lucy Newman under the direction and supervision of Jemma Franklin and Adam Blanchard.

To identify antimicrobial resistance genes, the genomes of 266 *S.* Dublin isolates and 266 *S.* Typhimurium from various origins of isolation in the UK were compared to the MEGARes 2.0 database containing all known AMR genes (Doster et al., 2020). Identity scores were generated for each AMR gene using the Basic Local Alignment Search Tool (BLAST) and an identity threshold of 90.0 was implemented to exclude dissimilar sequences. The AMR genes identified were grouped by antimicrobial class using information from the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020; McArthur et al., 2013) and MEGARes. Isolates were grouped into resistance profiles based on their differences in gene presence and absence and copy number.

6.2.3 - Statistical analysis

ANOVA and Kruskal-Wallis multiple comparisons were used to discern differences between groups of isolates based on their origins of

isolation. Mann Whitney U tests were used to compare the average number of resistance genes present in each isolate between the two serovars. Fisher's Exact tests were performed to identify significant differences between presence, vs absence in particular groups of isolates and resistance genes. Statistical analysis was performed using GraphPad Prism 8.1.

6.3 - Results

Antimicrobial resistance is a threat to both human and animal health. It is therefore important to understand existing AMR in the UK across a broad range of different isolates of *S.* Dublin and *S.* Typhimurium from various sources and identify potential for the acquisition or development of AMR to substances not currently routinely phenotypically tested.

6.3.1 - Antibiotic Sensitivity of *S.* Dublin isolates to antibiotics commonly used in cattle

Antibiotic sensitivity testing was performed on the 16 *S.* Dublin isolates from the APHA using six antibiotics to represent six antibiotic classes: streptomycin (aminoglycosides), tetracycline (tetracyclines), amoxicillin-clavulanic acid (β-lactams), nalidixic acid (quinolones), trimethoprim-sulfamethoxazole (sulphonamides) and chloramphenicol (phenicols). These antibiotics are representatives of classes commonly used in cattle based on UK sales data (Veterinary Medicines Directorate, 2016a).

All isolates were sensitive to all the antibiotics tested (Table 6.2). Intermediate sensitivity to streptomycin was observed in 14 of the 16 isolates, with the remaining 2 isolates being sensitive (Table 6.2).

Table 6.2 – Measurements of the zones of inhibition (ZOI) and interpretation of antibiotic resistance of *S.* Dublin isolates implicated in disease in cattle.

Antibiotic	Disc Quantity	Interpretive categories and ZOI diameter breakpoints (mm)			Number of isolates per category		
		Sen.	Int.	Res.	Sen.	Int.	Res.
Streptomycin	10µg	>15	12-14	<11	2	14	0
Tetracycline	30µg	>15	12-14	<11	16	0	0
Amoxicillin-Clavulanic acid	30µg	>18	14-17	<13	16	0	0
Nalidixic acid	30µg	>19	14-18	<13	16	0	0
Trimethoprim-sulfamethoxazole	25µg	>16	11-15	<10	16	0	0
Chloramphenicol	30µg	>18	13-17	<12	16	0	0

15 *S.* Dublin isolates from cases of bovine abortion in 2017 in the UK, and one laboratory strain were grown in nutrient broth overnight in a shaking incubator at 37°C. 100µl of these cultures was spread onto Mueller Hinton agar and antibiotic discs were placed onto the plates before incubation for 16h at 37°C. ZOIs were measured after incubation and the average of three experimental repeats is presented. Zone diameter breakpoints based on the Clinical and Laboratory Standards Institute (CLSI). n=3.

6.3.2 - Antimicrobial resistance genes in *S.* Dublin isolates associated with bovine abortion

Susceptibility testing isolates allows for a practical and clinically relevant evaluation of currently circulating isolates and their resistance. However, this method only accounts for the antibiotics in the testing panel and does not provide an insight into the progression of acquisition. Identifying AMR genes in the whole genome sequences aids in surveillance of this acquisition process and allows identification of resistance outside the usual panel of antibiotics tested, including biocides and metals.

A total of 33 antimicrobial resistance genes were identified in the *S.* Dublin isolates associated with bovine abortion from the APHA and laboratory isolate 2229. This included one antibiotic specific gene (*bacA* conveying resistance to bacitraicin), four genes conveying resistance to two different antibiotic classes (AAC6-PRIME and *kdpE* conveying resistance to aminoglycosides and *ampH* and PBP2

conveying resistance to beta-lactams), three multi-drug resistance genes (*msbA, sdiA* along with histone-like nucleoid structuring protein H-NS), thirteen multi-compound drug and biocide resistance genes (*acrA, acrB, bcr*, *cpxAR, crp*, *emrA, emrB, emrD, emrR, marA, marR, mdtK* and YOGI), one multi-compound drug and metal resistance gene (*pmrG*) and eleven multi-compound drug, biocide and metal resistance genes (*acrD, baeR, baeS, gesA, gesB, gesC, mdtA, mdtB, mdtC, robA* and *soxS*) (Table 6.3). All of the isolates harboured the same resistance genes, including two copies of biocide resistance genes *cpxAR* and *mdtK*.

Table 6.3 - Antimicrobial resistance genes identified in *S.* Dublin isolates associated with bovine abortion, grouped into antibiotics, antibiotic classes and multi-drug compartments. The whole genome sequences of 15 *S.* Dublin isolates from the APHA and laboratory strain 2229 isolated from calf salmonellosis were compared to the MEGAres database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020).

Antimicrobial Resistance	Total no. associated AMR genes in *S.* Dublin	AMR Genes	MEGARes Accession no.
Bacitraicin (antibiotic)	1	*bacA*	MEG_1189
Aminoglycosides	2	AAC6-PRIME	MEG_296
		kdpE	MEG_3448
Beta-lactams	2	*ampH*	MEG_729
		PBP2	MEG_5400
Multi-drug	3	H-NS	MEG_3271
		msbA	MEG_4061
		sdiA	MEG_6176
Multi-compound (drug and biocide)	13	*acrA*	MEG_399
		acrB	MEG_401
		bcr	MEG_1210
		cpxAR	MEG_2121
			MEG_2122
		crp	MEG_2132
		emrA	MEG_2721
		emrB	MEG_2725
		emrD	MEG_2729

		emrR	MEG_2734
		marA	MEG_3662
		marR	MEG_3663
		mdtK	MEG_3760
			MEG_3759
		YOGI	MEG_7842
Multi-compound (drug and metal)	1	*pmrG*	MEG_5802
Multi-compound (drug, biocide and metal)	11	*acrD*	MEG_ 404
		baeR	MEG_1191
		baeS	MEG_1194
		gesA	MEG_3132
		gesB	MEG_3133
		gesC	MEG_3134
		mdtA	MEG_3744
		mdtB	MEG_3748
		mdtC	MEG_3750
		robA	MEG_6082
		soxS	MEG_6551

6.3.3 - Antimicrobial resistance genes in 266 *S.* Dublin isolates from various sources

Understanding the antimicrobial resistance profiles of the 15 *S.* Dublin isolates from the APHA and laboratory strain 2229 was important in the context their use in other work as well as identifying resistance currently circulating in UK isolates. However, this was a relatively small sample size. Therefore, the whole genome sequences of 250 other isolates were downloaded from Enterobase to provide context for the 16 bovine isolates (Achtman et al., 2020; Alikhan et al., 2018). All 266 *S.* Dublin isolates in total have been described.

A total of 44 AMR genes were identified in the 266 isolates which included all of the AMR genes reported in the previous section as well as two genes associated with chloramphenicol resistance (*catA* and *floR*), one trimethoprim resistance gene (*dfrA*), three associated with aminoglycoside resistance (APH3-PRIME, APH3-DPRIME and APH6), three additional beta-lactam resistance genes (*cmy, ctx* and *tem*),

one sulphonamide resistance gene (*sulII*) and two tetracycline resistance genes (*tetA* and *tetC*) (Table 6.4). As in the previous analysis, two copies of the drug and biocide resistance genes *cpxAR* and *mdtK* were found in all isolates and all other genes were present in single copies.

Seven different resistance profiles (RPs) were identified in the 266 *S.* Dublin isolates (Figure 6.2). RP1 was shared by 259 isolates with various origins, including "livestock" (which included all of the previously analysed bovine abortion isolates) (n=104), "human" (n=123), "food" (n=22) and "other" (n=10). RP2 was identified in 2 isolates of "human" origin. RP4, RP6 and RP7 represented one isolate each, all of "human" origin. RP3 and RP5 represent one isolate of each of "livestock" origin.

Bacitracin resistance gene *bacA*, multi-drug resistance genes *mbsA* and *sdiA* along with histone-like nucleoid structuring protein H-NS (regulating the expression of a number of different efflux pumps), drug and metal resistance gene PMRG and 11 genes associated with drug, biocide and metal resistance (*acrD*, *baeR*, *baeS*, *gesA*, *gesB*, *gesC*, *mdtA*, *mdtB*, *mdtC*, *robA* and *soxS*) were identified in all isolates which was reflected in the resistance profiles (Figure 6.2). The presence of genes associated with resistance to chloramphenicol, trimethoprim, aminoglycosides, beta-lactams, drug and biocide compounds, sulphonamides and tetracyclines differed across the seven resistance profiles and were considered variable (Figure 6.2).

RP1 and RP3 were the most similar resistance profiles, differing only with multi-compound drug and biocide gene *emrA* being absent from RP3 which represented a "livestock" isolate (Figure 6.3). The "human" isolate represented by RP7 was the only isolate to have trimethoprim resistance gene *dfrA,* chloramphenicol resistance gene *catA* and beta-lactam resistance gene *ctx* (Figure 6.3). RP5 ("livestock" isolate) and RP6 ("human" isolate) were found to have a different chloramphenicol resistance gene, *floR* and like RP7, had sulphonamide resistance gene *sulII* and tetracycline resistance gene *tetA* (Figure 6.3). RP2 also had

tetA whilst RP4 was the only isolate to harbour tetracycline resistance gene *tetC* (Figure 6.3).

Genes conveying resistance to aminoglycosides and beta-lactams were variable among the different resistance profiles. Aminoglycoside resistance gene AAC6-PRIME was present in all but one isolate which was of human origin (RP4), whilst RP6 had five different resistance genes in this antibiotic group (AAC6-PRIME, APH3-DPRIME, APH3-PRIME, APH6 and *kdpE*) (Figure 6.3). RP2, 5 and 6 all had six aminoglycoside resistance genes, (AAC6-PRIME, APH3-DPRIME, APH6 and *kdpE*) (Figure 6.3). All isolates contained beta-lactam resistance genes *ampH* and PBP2 but only RP6 contained *cmy*, and RP7 contained *ctx* (Figure 6.3). Both RP2 and RP7 contained *tem* (Figure 6.3).

Table 6.4 - Antimicrobial resistance genes identified in *S.* Dublin isolates from various sources which were not previously identified, grouped into antibiotics, antibiotic classes and multi-drug compartments. The whole genome sequences of 266 *S.* Dublin isolates (250 from Enterobase, 15 from the APHA and laboratory strain 2229 isolated from calf salmonellosis) were compared to the MEGAres database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020). Genes identified in the previous analysis of APHA and laboratory *S.* Dublin isolates are not included in the table.

Antimicrobial Resistance	Total no. associated AMR genes in *S.* Dublin	AMR Genes	MEGARes Accession no.
Chloramphenicol (antibiotic)	2	*catA*	MEG_2132
		floR	MEG_2919
			MEG_2917
Trimethoprim (antibiotic)	1	*dfrA*	MEG_2517
Aminoglycosides	3	APH3-DPRIME APH3-PRIME	MEG_1019
			MEG_1079
		APH6	MEG_1084
			MEG_1086
Beta-lactams	3	*cmy*	MEG_1989
		ctx	MEG_2401
			MEG_2435
		tem	MEG_6875
			MEG_6909
Sulphonamides	1	*sulII*	MEG_6617
Tetracyclines	2	*tetA*	MEG_7024
			MEG_7025
		tetC	MEG_7065

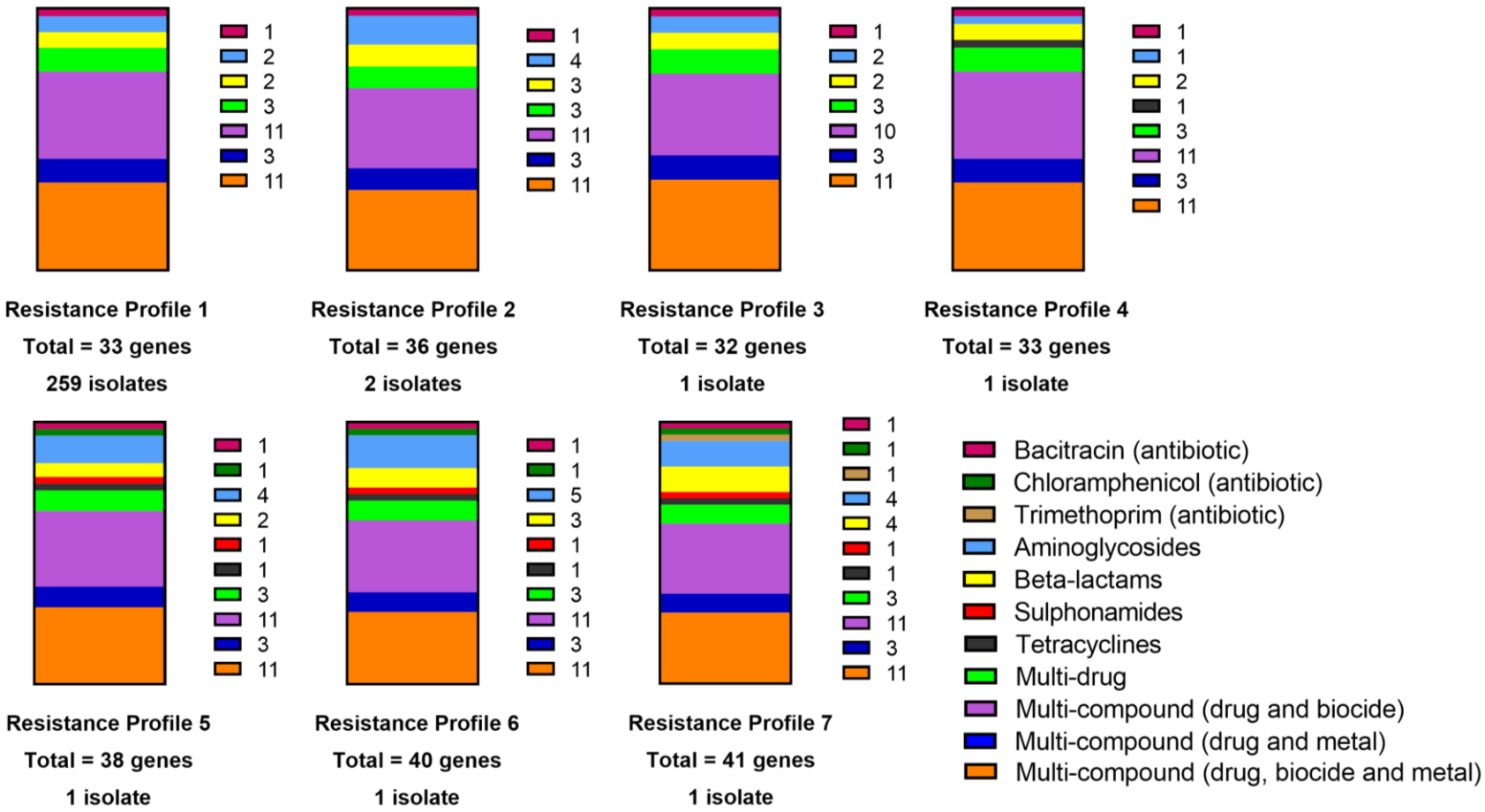


Figure 6.2 - Antimicrobial Resistance Profiles (RPs) of 266 *S.* Dublin isolates. The genomes of 266 *S.* Dublin isolates were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020) and grouped into profiles based on difference in gene presence and copy number. RP1 represents 259 isolates of origins including "livestock" (n=104), "human" (n=123), "food" (n=22) and "other" (n=10). RP2 represents 2 isolates of "human" origin. RP4, 6 and 7 represent one isolate each of "human" origin and RP3 and 5 represent one isolate each of "livestock" origin.

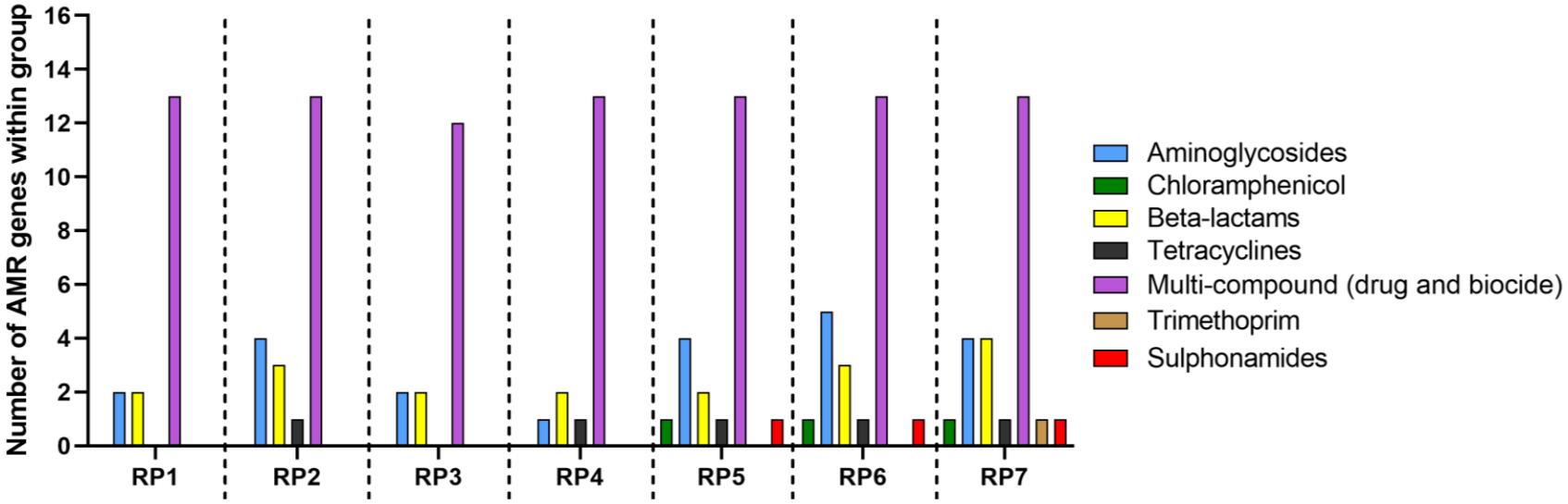


Figure 6.3 - Number of antimicrobial resistance genes present within variable groups across Resistance Profiles (RP) in 266 *S.* Dublin isolates. The genomes of 266 *S.* Dublin isolates were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020) and grouped into profiles based on difference in gene presence and copy number. "Variable groups" were defined as a group of antimicrobial resistance genes which were not found in the same frequency in all isolates or RPs. RP1 represents 259 isolates of origins including "livestock" (n=104), "human" (n=123), "food" (n=22) and "other" (n=10). RP2 represents 2 isolates of "human" origin. RP3, 5 and 6 represent one isolate each of "human" origin. RP3 represents 1 isolate of "bovine" origin. RP7 represents one isolate of "livestock" origin.

6.3.4 - Antimicrobial resistance genes in *S.* Typhimurium isolates

S. Typhimurium is more commonly associated with human gastroenteritis compared to *S.* Dublin and as such is likely to be exposed to a more varied range of antimicrobials. Similarly to *S.* Dublin however, surveillance of AMR in *S.* Typhimurium is limited. *S.* Typhimurium is capable of infecting cattle and humans and so could act as a reservoir for AMR acquisition for *S.* Dublin via horizontal gene transfer. Therefore, understanding the landscape of AMR in *S.* Typhimurium could aid in general surveillance and links back to *S.* Dublin and animal health.

A total of 71 antimicrobial resistance genes were identified in the 266 *S.* Typhimurium isolates downloaded from Enterobase, including genes conveying resistance to five specific antibiotics (bacitracin (n=1), chloramphenicol (n=2), fluoroquinolone (n=2), rifampin (n=1) and trimethoprim (n=1)), four antibiotic groups (aminoglycosides (n=9), beta-lactams (n=6), sulphonamides (n=3) and tetracyclines (n=6)), multi-drug (n=5) and macrolide, lincosamide and streotpgramine multi-drug (n=3), drug and biocide (n=14), drug and metal (n=1) and drug, biocide and metal resistance (n=11). Two copies of drug and biocide resistance gene *cpxAR* and drug and metal resistance gene *mdtK* were present in all isolates. Two copies of aminoglycoside resistance gene ANT3-DPRIME were present in nine isolates and aminoglycoside resistance gene APH3-DPRIME and beta-lactam resistance gene *ctx* were present in different individual isolates. One isolate had three copies of drug and metal resistance gene *mdtK*. All other genes were present in single copies. 30 of the 71 resistance genes were present in all isolates which included genes associated with resistance to bacitracin (*bacA*), beta-lactams (*pbp2*), multi-drug (*msbA* and *sidA,* as well as H-NS protein), drug and biocide (*acrA, acrB, bcr, cpxAR, crp, emrA, emrB, emrD, emrR, marA, marR* and *yogI*), drug and metal (*mdtK*) and drug, biocide and metals (*pmrG, baeR, baeS, gesA, gesB, gesC, mdtA, mdtB, robA* and *soxS*) (Figure 6.5). The other 41 genes were deemed

"variable". A further five genes were present in more than 95% of the isolates which included aminoglycoside resistance genes AAC6-PRIME, *acrD* and *kdpE,* beta-lactam resistance gene *ampH*, and drug, biocide and metal resistance gene *mdtC* (Figure 6.5).

The largest number of resistance genes identified in a single isolate was 47 (a single isolate associated with food) and the smallest number of genes in an isolate was 32, identified in isolates associated with food (n=1), livestock (n=1) and humans (n=4). When grouped into origins of isolation, the number of AMR genes identified differed significantly between isolates from human sources and isolates from livestock sources (P<0.0001) (Figure 6.4). The distribution of the number of genes in each isolate was similar in isolates of human, food and "other" origins, whilst the distribution of AMR genes from livestock associated isolates was considerably different (Figure 6.4). Chloramphenicol resistance gene *floR*, beta-lactam resistance gene *carB*, sulphonamide resistance gene *sulI,* tetracycline resistance gene *tetG* and aminoglycoside resistance gene ANT3-DPRIME were all identified significantly more often in livestock isolates compared to human isolates (Fisher's Exact test, P<0.0001). Two aminoglycoside resistance genes APH3-DPRIME and APH6, beta-lactam resistance gene *tem,* sulphonamide resistance gene *sulII* and tetracycline resistance gene *tetB* were identified significantly more often in human isolates compared to livestock isolates (Fisher's Exact test, P<0.0001).

57 RPs were identified among the 266 isolates, the largest profile representing 78 isolates and the smallest profiles representing a single isolate each (n=39) (Figure 6.5). Most of the isolates were accounted for in RPs 1-6, which contained ten or more isolates (n=188 of 266 isolates) (Figure 6.6). Isolates associated with livestock were represented by 14 different RPs, whilst human isolates fell into 30 different RPs (Figure 6.6). Food and "other" isolates were represented by 4 and 2 RPs respectively (Figure 6.6). RPs representing a single isolate included 26 human isolates, 10 bovine

isolates, one "other" isolate and two food isolates (Figure 6.6). Genes associated with resistance to chloramphenicol (*catA*), fluoroquinolone (*qnrB* and *qnrS*), rifampin (*arr)*, betal-lactams (*cmy* and *ctx*), tetracyclines (*tetC*, *tetD* and *tetM*), multi-drug (*oqxA* and *oqxB*), MLS (*mefB, mphA* and *mphB*) and drug and biocide (*cmlA*) compounds were found in very few isolates but accounted for a large amount of variation and resulted in the separation of many isolates into these single RPs.

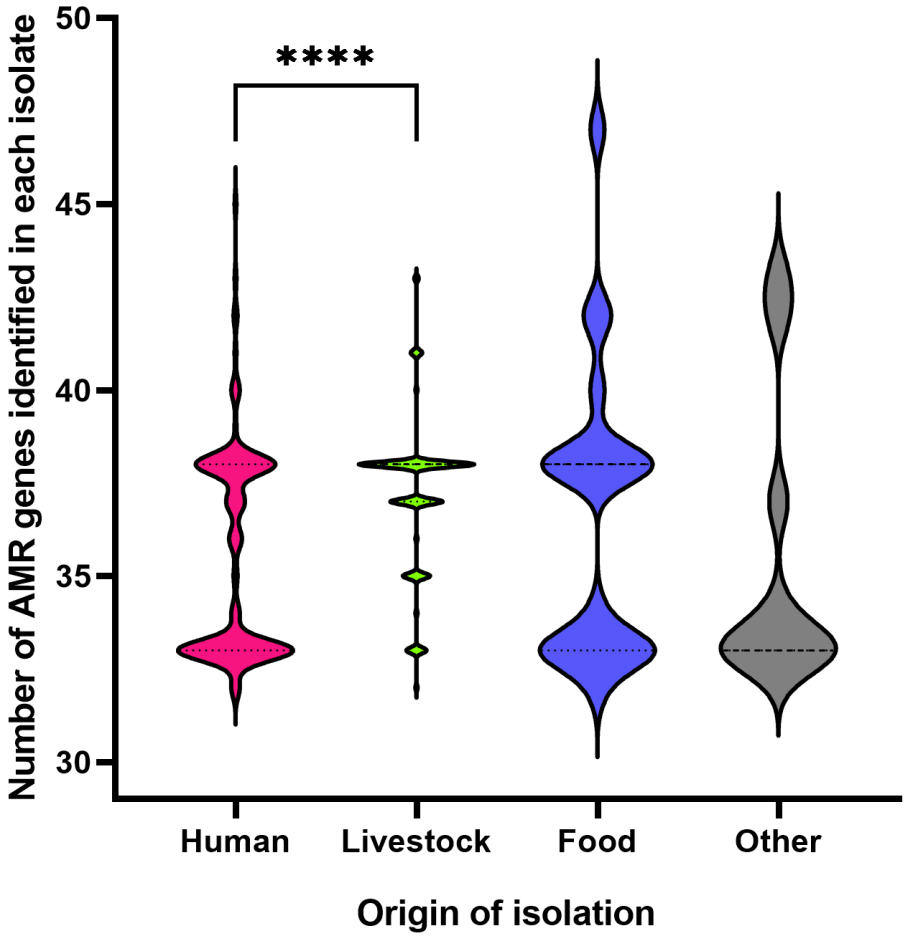


Figure 6.4 – Violin plots of the number of AMR genes identified in each *S.* Typhimurium isolate grouped by origin of isolation. The genomes of 266 *S.* Typhimurium isolates were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Evaluated using ANOVA and Kruskal-Wallis multiple comparisons test, P>0.0001. n=266

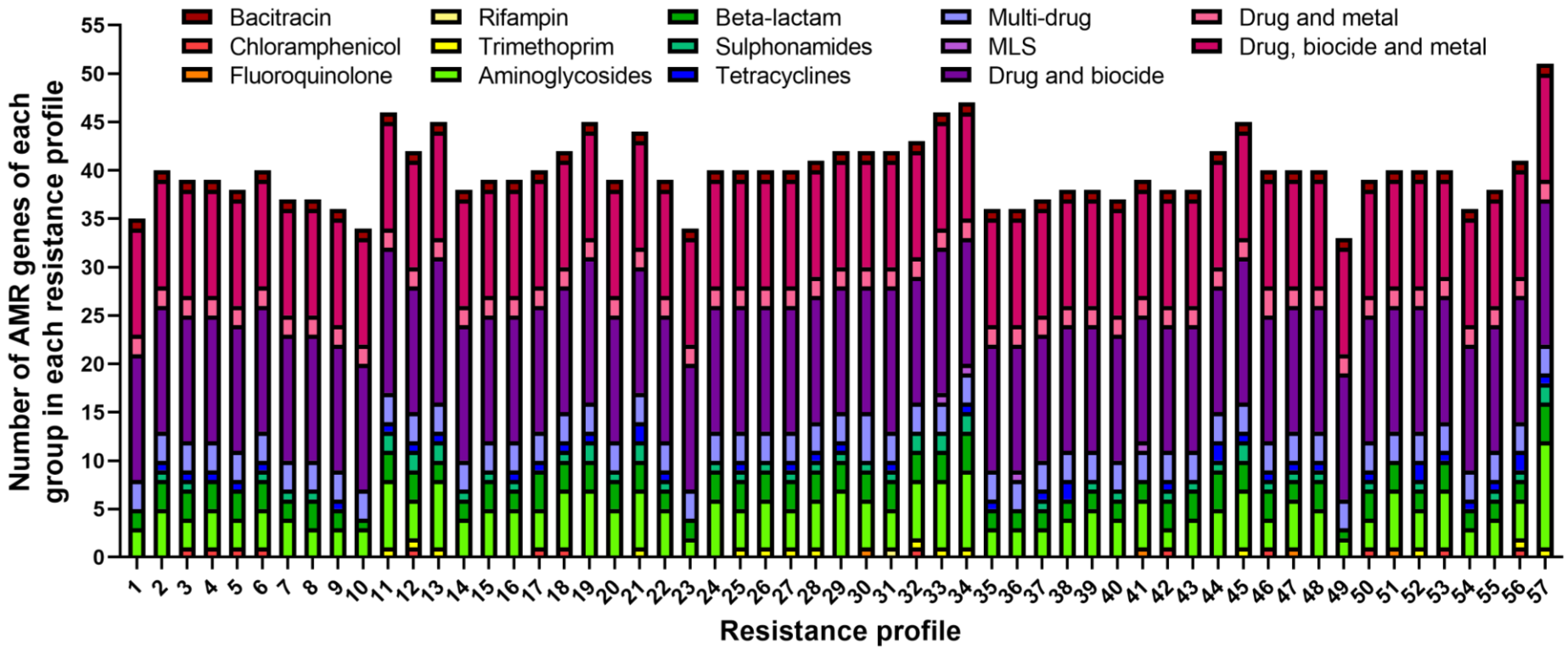


Figure 6.5 - Number of antimicrobial resistance genes within each group in resistance profile (RP) of *S.* Typhimurium isolates (n=266) from various origins of isolation. The genomes of 266 *S.* Typhimurium isolates were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020) and grouped into profiles based on differences in gene presence and copy number.

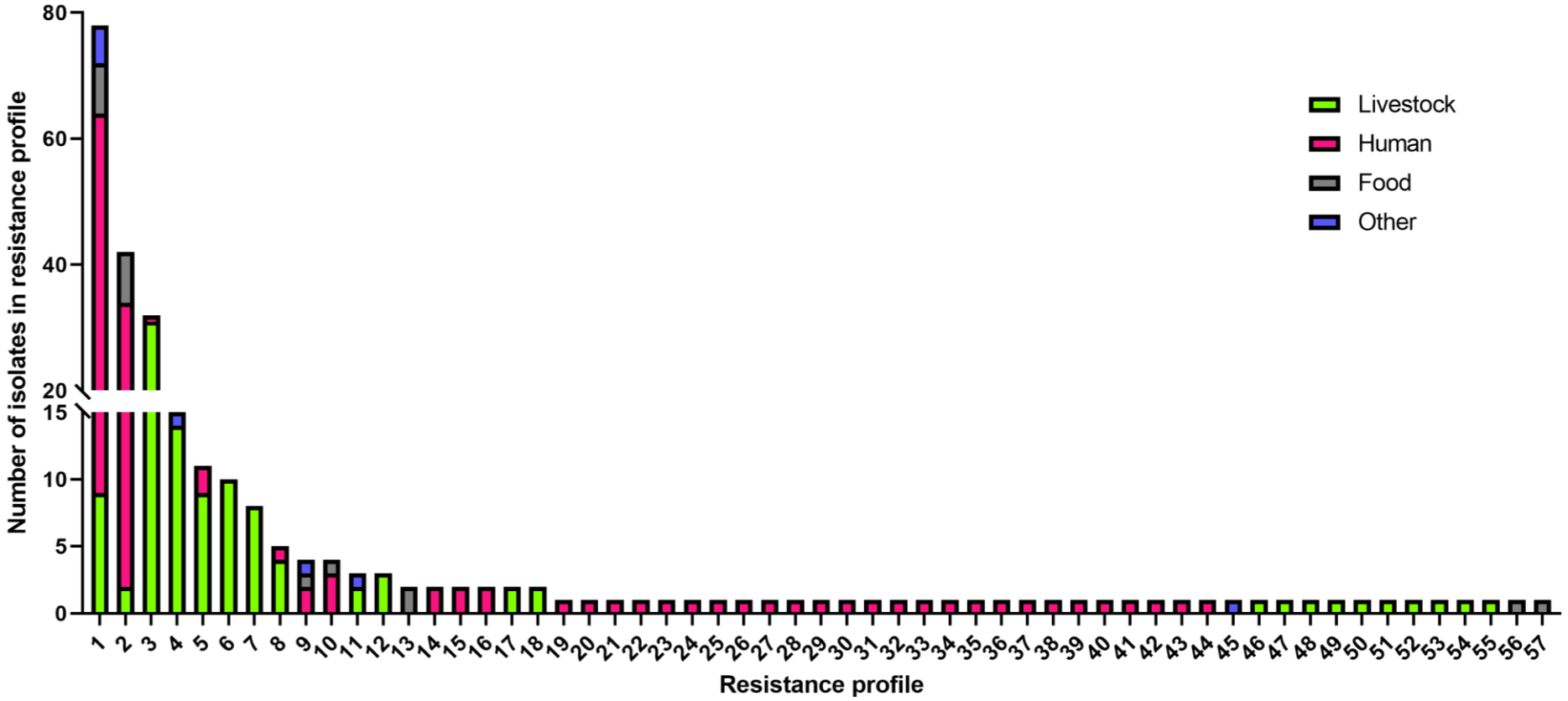


Figure 6.6 - Number of *S.* Typhimurium isolates in each antimicrobial resistance profiles. The whole genome sequences of 266 *S.* Typhimurium isolates were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial classes using information from CARD (Alcock et al., 2020) and grouped into profiles based on difference in gene presence and copy number. Isolates were grouped into "human" (n=128), "livestock" (n=106), "food" (n=22) and "other" (n=10).

6.3.5 - Comparison of antimicrobial resistance genes in *S.* Dublin to those in *S.* Typhimurium from similar origins of isolation

A larger number of AMR genes were identified in *S.* Typhimurium isolates (n=78) compared to *S.* Dublin (n=45) and *S.* Typhimurium tended to have more AMR genes on average compared to *S.* Dublin isolates (Mann Whitney U test, P<0.0001) (Figure 6.7). 19 genes were identified in *S.* Typhimurium which were not identified in *S.* Dublin which included resistance to fluoroquinolones (*qnrB* and *qnrS*), rifampin (*arr*), aminoglycosides (AAC3 and APH4), beta-lactams (*carB*), sulphonamides (*sulI* and *sulIII*), tetracyclines (*tetB, tetD, tetG* and *tetM*), multi-drug (*oqxA* and *oqxB*), and drug and biocide (*cmlA* and *qacL*). A new AMR gene group was also identified in Typhimurium which was not present in Dublin – MLS (*mefB, mphA* and *mphB*). The only gene present in Dublin and not Typhimurium was *acrD*, a gene associated with drug, biocide and metal resistance. A panel of 33 different AMR genes were identified in more than 99% of the *Salmonella* isolates of both serovars which could be considered "core" AMR genes. These included genes associated with resistance to bacitracin (*bacA*), aminoglycosides (AAC6-PRIME and *kdpE*), beta-lactams (*ampH* and PBP2), multi-drug compounds (*msbA* and *sdiA* along with H-NS protein), drug and biocide (*acrA, acrB, bcr,* one copy of *cpxAR*, *crp, emrA, emrB, emrD, emrR, marA, marR* and YOGI), drug and metals (*pmrG* and two copies of *mdtK*) and drug, biocide and metals (*baeR, baeS, gesA, gesB, gesC, mdtA, mdtB, mdtC, robA* and *soxS*).

There were no resistance profiles shared between any of the isolates in the two serovars because there were more "core" resistance genes present in *S.* Typhimurium isolates compared to *S.* Dublin isolates. *S.* Typhimurium isolates fell into considerably more resistance profiles compared to *S.* Dublin isolates due to the larger number of genes identified in *S.* Typhimurium isolates.

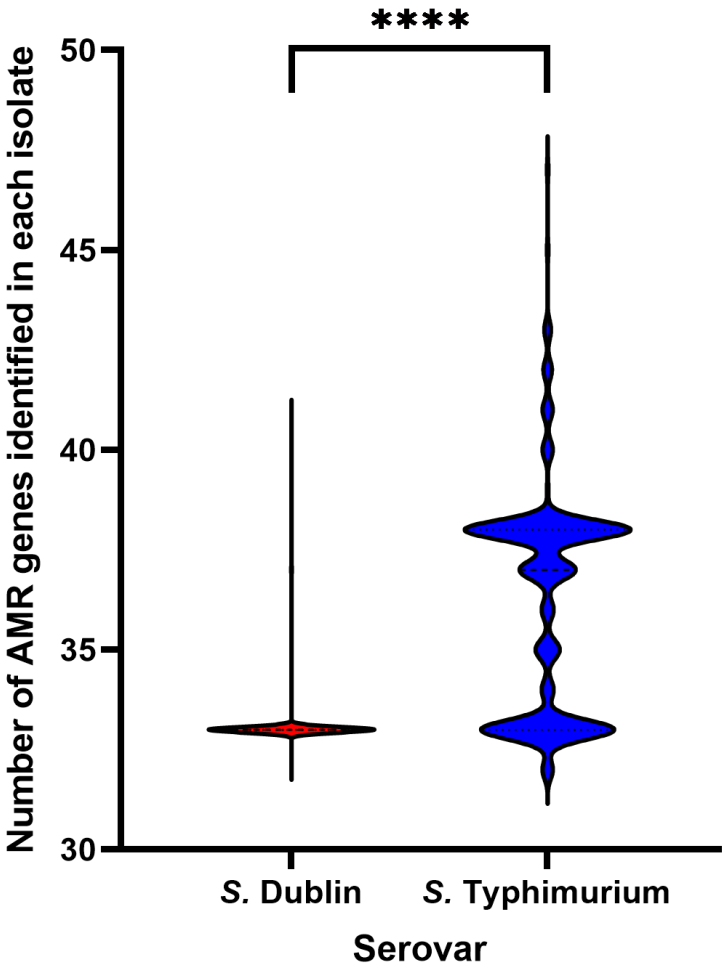


Figure 6.7 - Violin plots of the number of AMR genes identified in each isolate of 266 *S.* Dublin and 266 *S.* Typhimurium isolates. serovar. The genomes of 266 *S.* Dublin and 266 *S.* Typhimurium isolates were compared to the MEGARes database containing all known AMR genes, with an identify threshold of 90.0% (Doster et al., 2020). Evaluated using Mann Whitney U test, P>0.0001. n=532 isolates.

6.4 - Discussion

Antimicrobial resistance is a growing concern worldwide. Whilst antibiotics are infrequently prescribed in the UK for gastroenteritis and antibiotic usage is decreasing in livestock, it is still important that antimicrobial resistance is monitored over time (National Institute for Health and Care Excellence, 2022; Veterinary Medicines Directorate, 2019).

Antibiotic resistance in *S.* Dublin in the UK is relatively uncommon according to the yearly reports published by the Medicines

Directorate, with less than 5% of isolates tested being resistant to any of the antibiotics routinely used in treating clinical infections and tested for surveillance. In 2017, 272 isolates from various origins, including cattle, sheep and dogs were tested for resistance to ampicillin, chloramphenicol, nalidixic acid, streptomycin, tetracycline and trimethoprim/sulphamethoxazole which were included in this study, as well as furazolidone, neomycin and sulphonamide compounds (Veterinary Medicines Directorate, 2017). All the isolates from 2017 were susceptible to all the antibiotics tested (Veterinary Medicines Directorate, 2017). Intermediate resistance to streptomycin (an antibiotic in the aminoglycoside class) observed in 14 out of 16 isolates tested in this study cannot be compared to the surveillance reports from the Veterinary Medicines Directorate because intermediate resistance is not reported in the UK. However, resistance in *S.* Dublin has been reported in previous years (2014: 2.5% of isolates were resistant; 2015: 4%; 2016: 1.6%) and streptomycin resistance was observed in 13.9% of *Salmonella* spp isolated from cattle in 2018 (Veterinary Medicines Directorate, 2017, 2018). Aminoglycosides were some of the most commonly used antibiotics in both dairy (20%) and beef (18%) production in 2018, so it is logical that higher levels of resistance, (intermediate or otherwise) would be observed in this antibiotic class (Veterinary Medicines Directorate, 2018). Chromosomally encoded aminoglycoside resistance genes have been reported in *Salmonella* which can be silenced through deletions in the promoter region (McMillan et al., 2019). Such deletions may also explain the poor concordance between the AMR genes identified in the 16 *S.* Dublin isolates compared to their phenotypic resistance.

There was little variation amongst the 266 isolates genotypically analysed in terms of their antimicrobial resistance genes, with only seven profiles being found. This demonstrates the limited variation across a period of 18 years from various sources including livestock and humans in the UK. Of the genes identified, genes associated with resistance to aminoglycosides and beta-lactams were most often

observed. This is concerning, particularly for aminoglycoside resistance as this class of antibiotics is thought to be commonly used in livestock in the UK (Veterinary Medicines Directorate, 2018).

Multi-compound antimicrobial resistance genes were the most abundant genes identified across the *S.* Dublin isolates and can convey a range of different resistances to drugs, biocides and metals. Multi-compound genes *baeSR* and *cpxAR* were present in all *S.* Dublin isolates, both which induce MdtABC to facilitate the resistance of bacteria harbouring these gene clusters to novobiocin (an aminocoumarin), kanamycin (an aminoglycoside) and deoxycholate (a bile salt with antimicrobial properties) (Baranova and Nikaido, 2002; Hirakawa et al., 2003). The isolates used in phenotypic susceptibility testing harboured all three of these gene clusters but resistance was not evident because none of the antimicrobial compounds for which these genes convey resistance to were tested specifically. Similarly, all isolates including those tested for susceptibility harboured resistance gene cluster *gesABC* which can also be induced by CpxAR and can confer resistance to a wide range of antibiotic classes including beta-lactams (cefoxitin, cephalothin, cloxacillin, oxacillin and nafcillin), aminoglycosides (amikacin) and amphenicols (chloramphenicol and thiamphenicol) (Cerminati et al., 2017; Conroy et al., 2010). It is interesting that despite harbouring genes associated with resistance to chloramphenicol, none of the isolates tested were found to be resistant and implies a more complex regulatory mechanism controls resistance in these isolates than only *gesABC*. Similarly, gene cluster *acrAB* was also present in all of the isolates and is regulated by *soxS, robA* and *marA*, all of which were identified in *S.* Dublin (Giraud et al., 2000; Nikaido et al., 1998; Okusu, Ma, and Nikaido, 1996; White et al., 1997). This regulatory network and expression of AcrAB is associated with resistance to a range of antimicrobials, including tetracycline, nalidixic acid and chloramphenicol which was not observed in any of the isolates tested (Usui et al., 2013). Two copies of *mdtK* were identified in all of the *S.* Dublin isolates which are associated with resistance to norfloxacin (a

quinolone), doxorubicin (an antitumour antibiotic) and acriflavine (an antiseptic) in *S.* Typhimurium isolates (Nishino, Latifi, and Groisman, 2006). Gene cluster *emrAB* can confer resistance to novobiocin, nalidixic acid, rhodamine 6G (a phosphate salt) and sodium-deoxycholate in *S.* Typhimurium (Nishino, Latifi, and Groisman, 2006). Many of the genes and associated resistances which they convey are not part of the usual process of susceptibility testing in routine surveillance which could hinder effective surveillance. However, as demonstrated here, the presence of genes conveying resistance to nalidixic acid and tetracycline as well as chloramphenicol does not ensure that isolates will be resistant. Nevertheless, monitoring resistance and emphasising responsible use of antibiotics is of the utmost importance, exemplified by *S.* Dublin resistance in the United States where this serovar is one of the most multi-drug resistant *salmonellae* (Cummings et al., 2019; Srednik et al., 2021).

Antimicrobial resistance genes were more abundant in *S.* Typhimurium isolates in this data set compared to *S.* Dublin, in keeping with comparative general trends of resistance in the UK. In the UK in 2017, human isolates were found to be non-susceptible to ampicillin (53%), streptomycin (49%), gentamycin (66%), sulphonamides (56%), tetracycline (63%), cefotaxime (4%), ceftazidime (7%) and ciprofloxacin (10%) (Veterinary Medicines Directorate, 2019). A proportion of isolates tested were also non-susceptible to chloramphenicol (percentage not detailed) (Veterinary Medicines Directorate, 2019). In livestock isolates (including cattle, pigs and chickens), *S.* Typhimurium isolates were resistant to nalidixic acid (2%), chloramphenicol (78%), trimethoprim/sulphonamide (33%), ampicillin (80%), streptomycin (75%), tetracycline (73%) and sulphonamide compounds (86%) (Veterinary Medicines Directorate, 2019). It was therefore anticipated that the majority of isolates isolated from livestock sources in this study had chloramphenicol resistance gene *floR*. Whilst there is no data available for these isolates in terms of their phenotypic

resistance, the high incidence of *floR* indicates a potential for resistance in these isolates.

This widespread resistance is concerning because chloramphenicol is a highly important antibiotic in human medicine and health (World Health Organisation, 2018). This increasing resistance in both human and animal isolates reduces the potential use for this antibiotic. Genes associated with resistance to tetracycline antibiotics were also widespread, with at least one resistance gene present in 152 of the *S.* Typhimurium isolates. With phenotypic resistance identified in both human and animal associated isolates in the UK in 2017, this is again expected (Veterinary Medicines Directorate, 2019). Furthermore, tetracyclines are some of the most frequently used antibiotics in UK livestock medicine so selection pressure will be high in livestock reservoirs (Veterinary Medicines Directorate, 2016b). Similar to chloramphenicol, tetracycline is classed as a highly important antimicrobial in human health by the World Health Organisation, so increasing resistance is of concern for both human and animal health (World Health Organisation, 2018). Streptomycin and gentamycin resistance is observed in livestock isolates in the UK and gentamycin resistance is infrequently observed in human *S.* Typhimurium isolates in the UK (Veterinary Medicines Directorate, 2019). Genes associated with resistance to aminoglycosides were widespread in the *S.* Typhimurium isolates evaluated here, with three genes present in more than 95% of the isolates. Furthermore, genes associated with resistance to aminoglycosides were the third largest group of antimicrobial resistance genes identified (n=8).

Similar to the *S.* Dublin isolates characterised, *S.* Typhimurium isolates harboured a considerable number of multi-compound resistance genes, the majority of which were also present in *S.* Dublin. An additional macrolide resistance cluster was identified in the MLS group, *mphAB* but these genes were not present together in any isolates and were only present in three isolates in total. It is thought that *mphAB* is one of the main drivers of resistance to

azithromycin which is classed as a critically important antibiotic by the World Health Organisation and as such should be carefully monitored (Gomes et al., 2019; World Health Organisation, 2018; Xiang et al., 2020). Additionally, *mefB* was identified in another isolate which also is associated with macrolide resistance (Liu et al., 2009). These macrolide resistance genes were all identified in human isolates where it is more likely that macrolides like azithromycin would be used on the rare occasion that intervention in a clinical case of gastroenteritis would occur (Veterinary Medicines Directorate, 2019).

There was considerably more variation among *S.* Typhimurium human isolates compared to livestock isolates, exemplified by human isolates falling into more than twice as many resistance profiles despite having only 22 more isolates in this category. This could be due to the differences in antibiotic use in human and animal medicine, where isolates in humans may face different selection pressures to a wider range of antibiotics compared to livestock.

In the US, certain plasmids harbouring AMR genes are more often associated with host-adapted isolates from cattle than any other reservoir, demonstrating the potential for fundamental differences in antimicrobial potential between serovars and isolates from certain hosts (McMillan et al., 2019). It is unclear as to why this may be the case but could be due to differences in antimicrobial usage in human versus animal medicine or due to differences in hosts creating environmental niches which aid in facilitating horizontal gene transfer. The gastrointestinal tracts of both humans and ruminants are known to have their own unique and heritable microbiomes and resistomes (Auffret et al., 2017; Grieneisen et al., 2021; Li et al., 2019; Penders et al., 2013). Therefore, host adapted serovars like Dublin may be more likely to encounter a particular subset of AMR genes compared to host generalists like Typhimurium which could encounter larger subsets of AMR genes.

The use of susceptibility testing is useful in a clinical setting as it provides insight into what antibiotics would be appropriate to use. However, phenotypic susceptibility testing only provides evidence of resistance for the specific antibiotics tested which can differ between antibiotics in the same class and has been shown in *S.* Dublin isolates (Srednik et al., 2021). Moreover, the antibiotics used in this study were chosen based on sales data because usage data in animals is not currently available. Therefore, whilst the antibiotic panel was chosen based on available data, it may not have been entirely representative of what is actually used in livestock in the UK. The identification of AMR genes was also limited in that it was not possible to identify the context in which these genes are found.

The action of antimicrobial resistance genes will rely on the expression of regulatory genes and elements like pili which were not accounted for within this study because many are not included in the database of resistance genes used. For example, histone-like nucleoid-structuring protein H-NS was identified in all *S.* Dublin and *S.* Typhimurium isolates and is a global regulatory factor involved in the expression of different efflux pumps The database fortuitously included H-NS but this was not deliberately sought out. It would therefore be beneficial to identify the regions around the identified resistance genes to discern if these genes could be functionally expressed. In this sense, using a tool like PlasmidFinder could be beneficial as this would aid in characterising mobile genetic elements associated with AMR and virulence which in turn would aid in our understanding of the impact of mobile genetic elements on AMR in *Salmonellae* in the UK (Carattoli et al., 2014). Additionally, studies have shown that pseudogenisation of AMR genes can occur when the bacteria harbouring these genes are not challenged with antibiotics, including on farm (Davis et al., 2011). It may be that, due to the push to reduce antimicrobial usage in livestock in the UK, pseudogenisation of the genes present in these isolates may have occurred which has impacted their functionality in conveying phenotypic resistance (Veterinary Medicines Directorate, 2020). However, as many of the

genes present in this study had sequence identities of 100%, it is unclear as to whether this is the case.

6.5 - Summary

The susceptibility of the *S.* Dublin isolates tested in this work is in keeping with current trends in AMR in this serovar in the UK. However, the identification of AMR genes in these and a wider pool of *S.* Dublin isolates demonstrates the potential for the acquisition of antimicrobial resistance genes through horizontal gene transfer which could eventually become phenotypic resistances. Similarly, AMR genes in *S.* Typhimurium correspond well with currently available surveillance data on phenotypic resistance but exemplify the potential for AMR acquisition in the future. Differences in AMR genes between serovars was to be expected, though differences in AMR genes between origins of isolation demonstrates how the host environment and human use of antibiotics could impact the same serovar in different hosts. Surveillance of AMR genes once an organism has been isolated could aid in our understanding of the progression of AMR acquisition between different hosts and serovars and could inform our use of antibiotics in certain clinical situations in addition to susceptibility testing.

Chapter 7 - General Discussion

The overarching aim of the work presented in this thesis was to improve understanding of *S.* Dublin as a causative agent of abortion in cattle. This included understanding more about the virulence of *S.* Dublin, how this might impact the progression of disease and the host response to the disease as an underlying mechanism of abortion events. As research into *S.* Dublin generally is relatively limited and research into how and why *S.* Dublin causes abortion is scarce, much of the work presented here can be used as a foundation for further work.

7.1 - Investigation of virulence genes present in *S.* Dublin isolates requires further validation

As a comparatively under-researched serovar, investigating the virulence factors present in *S.* Dublin was a necessary step to improve the understanding of this organism. However, interpreting the results obtained from the virulence factor studies in isolation proved challenging because of this lack of research on *S.* Dublin. Comparing the virulence factors of *S.* Dublin to those of *S.* Typhimurium, a considerably more researched broad-host range serovar, was a logical foundation upon which to understand *S.* Dublin virulence results. Instead, these studies highlighted a number of limitations in using the VFDB (Liu et al., 2019).

Three examples of seemingly non-sensical results identified in the work presenter here were particularly noteworthy, the first being the apparent "absence" of essential SPI2 T3SS component *ssaU* sequences from human-associated and presumably virulent *S.* Dublin isolates. A number of groups have shown that SsaU is essential for the functionality of the SPI2 T3SS, without which *Salmonella* isolates

become essentially avirulent (Cox et al., 2016; Feria et al., 2015; Riordan and Schneewind, 2008; Sabag-Daigle et al., 2016; Shea et al., 1996; Sorg et al., 2007; Valdivia and Falkow, 1997; Yu et al., 2018). Other groups have analysed *S.* Typhimurium fimbrial components in depth and shown that virulent *S.* Typhimurium isolates possess a complement of fimbrial operons similar to those identified in *S.* Dublin (Yue et al., 2012). These were not identified in presumably virulent *S.* Typhimurium isolates analysed in this study. Finally, the "absence" of genes associated with key ferric uptake processes in *S.* Dublin and *S.* Typhimurium isolates demonstrated that there were numerous results generated using the VFDB that were unreliable (Nagy et al., 2013). A host of researchers have used the VFDB to investigate *Salmonella*, including groups working within government bodies like the APHA in the UK, with very few pointing out flaws in its usage (Kirkwood et al., 2021). However, a preprint paper described there being a notable absence of some ferric-uptake related genes, and another described there being a notable lack of several important SPI1 (*sopD2* and *sopA*) and SPI2 (*sssU, ratB, steC* and *sseK1*) virulence genes which was unexpected (Darboe et al., 2020, 2022; dos Santos et al., 2021). Along with the findings presented in this work, these papers highlight the requirement for validation of any results generated using the VFDB.

Some of the factors which could be impacting the validity of the results gleaned from the VFDB could include whether the database contains records of genes from specific serovars, especially when host-specific niches are concerned. Even when there are serovar-specific records available, these records can pertain to isolates from almost eighty years ago where genetic drift could then impact the likelihood of comparisons producing accurate presence/absence results (Lilleengen, 1948). Geography and time could both influence the outcome of studies using the VFDB because of genetic drift, where bacterial lineages diverge and genes are no longer identical. Some of these factors could be overcome using different identity thresholds to obtain presence/absence results. There does not appear to be a

consensus in the literature of an acceptable threshold to use where some groups have utilised 70% whilst others use up to 95% (González-Torres et al., 2023; Seribelli et al., 2021; Wang et al., 2022). This flexibility may also aid in the use of WGSs of sub-optimal quality or coverage which, whilst unideal, is important for investigating serovars with few records or isolates of importance. However, these results should be validated using alternative methods because of the issues outlined here.

Methods of validating results obtained using the VFDB could include using alternative bioinformatic approaches. For example, isolating the sequences of genes of interest once they have been identified and querying them in NCBI BLAST against other sequences that have been uploaded to this database instead of the VFDB may be useful (Altschul et al., 1990). Amino acid sequences and predicted proteins could be investigated using programmes like Jalview and EMBOSS (Rice, Longden, and Bleasby, 2000; Waterhouse et al., 2009). There are also alternative databases specifically for bacterial virulence factors which have been developed and could be used instead of or alongside the VFDB. Examples include the Victors database and the PATRIC bacterial bioinformatics database which incorporates information from both Victors and the VFDB (Sayers et al., 2019; Wattam et al., 2014). Using well-annotated and previously studied genomes, the VFDB and other virulence factor-focussed databases and tools could be validated where the results can be more accurately predicted.

To validate the results obtained in the studies presented here, it may be useful to analyse the *S.* Dublin and *S.* Typhimurium isolates using the PATRIC bacteria bioinformatics database as this compares information from both the VFDB and the Victors database (Liu et al., 2019; Sayers et al., 2019; Wattam et al., 2014). This should mean that the isolates are compared to a larger range of records as the work here which should increase the reliability of any outcomes. It also means that any updates and improvements to the VFDB since

the time of analysis can be included and impact the results (Liu et al., 2022). As the algorithms used by the VFDB are not well described, further validation and analysis of any genes of interest using bioinformatic techniques as described above as well as laboratory techniques like PCR to confirm the findings would be beneficial.

7.2 - Phenotypic virulence of *S.* Dublin in host placental tissues

Experimental infection of pregnant cattle and calves with *S.* Dublin has been conducted which describe the presence of the causative bacteria localised to various organs (Hall and Jones, 1977; Pullinger et al., 2007; Vohra et al., 2019). The virulence of *S.* Dublin isolates have been explored in a limited range of tissues from various hosts, with the most host and tissue specific studies relevant to bovine infection and abortion conducted in bovine ileal loop models (Pullinger et al., 2007; Vohra et al., 2019). *S.* Dublin has been isolated from the placentome during experimental infection of pregnant cattle and studies into the host response to heat-killed microorganisms have been conducted using bovine intercotyledonary and endometrial tissues and *S.* Dublin (Hall and Jones, 1977; Hall et al., 1979; Silva et al., 2012). However, until now, no studies have investigated the invasion and survival of live *S*. Dublin in reproductive tissues. MOIs of 1 and 10 were observed to invade and replicate in BCECs in a dose-independent manner without killing the host cells. The use of a gentamycin protection assay-like protocol determined that the bacteria recovered from these cultures were intracellular, showing for the first time the presence of intracellular *S.* Dublin in placental tissues. Further work using a more complex model of the reproductive tract would be beneficial as it is possible that the different cell types present in the placentome are differentially susceptible to invasion by *S.* Dublin, as has been observed in BCECs and F3 fetal cells infected with *Neospora caninum* (Jiménez-Pelayo et al., 2019a). This would aid in understanding of how the placenta is colonised and could include investigation into the progression of fetal colonisation and

death as observed in pregnant animals experimentally infected with *S.* Dublin (Hall and Jones, 1977). During pilot studies conducted with BCECs and *S.* Dublin, MOIs of more than 10 resulted in considerable cell death but was not investigated further as quantifying the virulence of the bacteria specifically would have been considerably more difficult. However, this implies that infection with *S.* Dublin can lead to the death of BCECs, which could be highly detrimental in the placentome. The de-lamination of the caruncular and cotyledonary structures in the placenta due of tissue death would likely prevent nutrient exchange and lead to fetal death (Longbottom and Coulter, 2003).

7.3 - Survival of *S.* Dublin in bovine blood and routes of dissemination

S. Dublin are able to infect bovine macrophages, supporting the general theory that *Salmonellae* could be disseminated systemically inside host phagocytes (Qureshi, Templeton, and Adams, 1996; Rice, Besser, and Hancock, 1997; Vohra et al., 2019). Other work has suggested that free-living bacteria are disseminated via the lymphatic system, though the sampling regime was limited to organs like mesenteric lymph nodes, spleen and liver and did not explore further systemic dissemination (Pullinger et al., 2007). The lymphatic system drains away from the gastrointestinal tract and mesenteric lymph nodes for filtering and re-circulation as a component of blood, so it seems unlikely that bacteria would be disseminated from these locations towards the reproductive tract. Additionally, there does not appear to be citation of bovine lymphatic vessels existing in the placentome anywhere in the literature. Research has ascertained that lymphatic vessels are not present in the human placenta so it is possible that this is also the case in cattle (Becker et al., 2020; Castro, Parks, and Galambos, 2011). *S.* Dublin bacteraemia has been reported in various cases, leading to the possibility that the bacterium could be disseminated in the blood (Nielsen, 2013a). Blood from the gastrointestinal tract passes through the liver where infectious foci

have been identified during experimental infection of pregnant cattle with *S.* Dublin (Hall and Jones, 1977). In these pregnant animals infected with *S.* Dublin, it was hypothesised that infection of the liver, spleen and lymph nodes occurred initially, followed by infection of reproductive tissues approximately a week after inoculation (Hall and Jones, 1977). Given that *S.* Dublin was shown to survive in the blood in this study and others, systemic dissemination from the liver towards the reproductive tract now seems like an alternative or additional explanation to the infection of monocytes and macrophages, or via the lymphatic system (Pullinger et al., 2007). Furthermore, the highly convoluted vasculature of the placentome would produce eddies in blood flow, similar to those in the spine of dogs where bacteraemia leads to diskospondylitis, which allow bacteria to adhere to and infect endothelial cells and enter other tissues (Betbeze and McLaughlin, 2002; Haeger, Hambruch, and Pfarrer, 2016; Schlafer, Fisher, and Davies, 2000).

A method of further investigating the possibility of free-living bacteria disseminating to the placentome and causing infection could include using an *ex vivo* organ culture model of the placentome. Flushing an extracted placentome via the afferent artery with different concentrations of *S.* Dublin harbouring a green-fluorescent protein (GFP) reporter plasmid and subsequent histological analysis could demonstrate whether eddies in blood flow impact bacterial adherence and contribute to understanding of dissemination. Additionally, investigation into the presence of lymphatic vessels present in the bovine placentome could contribute to the discussion of the involvement of lymphatic dissemination. This could be achieved by histological examination of the placentomes and using immunohistochemistry to stain the endothelial cells of the lymphatic vessels. Investigation of lymphatic vessels has been conducted in the corpus luteum of pregnant animals using the lymphatic endothelial hyaluronan receptor 1 (LYVE1) (Nitta et al., 2011). It is possible that invasion of the lymphatic system is essential for initial dissemination from the intestine to the liver and thereafter the bacteria become

blood-borne and access the reproductive tract. Knowing whether the involvement of lymphatic dissemination to the placenta is possible can inform future work into *S.* Dublin-induced abortion and other reproductive diseases.

7.4 - The host immune response elicited by *S.* Dublin

Like many other facets of *S.* Dublin virulence and infection, the host response to *S.* Dublin is poorly characterised, and is especially poorly characterised in the context of the pregnant bovine reproductive tract. As neutrophilic infiltration of the placenta is observed in abortion events associated with *S.* Dublin (alongside other infections including *Listeria monocytogenes* and *Brucella abortus*), a logical choice for characterisation of the immune response in the reproductive tract is the neutrophil chemokine CXCL8. The expression of CXCL8 in intercotyledonary and endometrial tissues in response to heat-killed *S.* Dublin has been evaluated, where CXCL8 transcription was significantly elevated in stimulated versus unstimulated tissues (Silva et al., 2012). This demonstrated that an immune response is elicited in the placentome due to the presence of heat-killed *S.* Dublin. However, *Salmonellae* have various mechanisms of immune evasion which cannot be evaluated using heat-inactivated microorganisms. Furthermore, the expression of cytokines or chemokines besides CXCL8 which could be associated with *S.* Dublin infection have been evaluated in bovine reproductive tissues prior to the work presented in this thesis.

The upregulated expression of CXCL8 and TNFα in response to *S.* Dublin infection of BCECs observed in this study is similar to the immune responses observed to abortifacient pathogens like *Neospora caninum, Chlamydia abortus* and *Listeria monocytogenes* (Leaver et al., 1989; Rosbottom et al., 2008; Wheelhouse et al., 2009). Upregulation of CXCL8 in the placentome correlates with the increase in neutrophils observed during placental infection and subsequent abortion, whilst necrotic foci could be a result of TNFα expression triggering apoptosis (Hall and Jones, 1977). However, the action of

neutrophils present or the infection and lysis of host cells by the invading bacteria could also cause tissue necrosis (Rydell-Törmänen, Uller, and Erjefält, 2006). These mechanisms are also complicated by the presence of PGE_2 which interacts with immune cells to alter their inflammatory phenotype, and unfortunately could not be characterised in the studies presented here (Pitts and D'Orazio, 2019). Whilst demonstration of the upregulation of these proinflammatory mediators is an important first step into understanding the host response during *S.* Dublin infection, there are a number of other factors to consider.

The Th2 bias includes the presence of anti-inflammatory mediators which were not investigated in this work. For example, IL-10 is associated with successful pregnancy, but currently there is no information available about the impact of *S.* Dublin on IL-10 regulation and the downstream impact on pregnancy (Oliveira et al., 2013). Previous work characterising the immune response to *Neospora caninum* in BCECs did not detect IL-10 so it is possible that this cell line does not produce the anti-inflammatory cytokine (Jiménez-Pelayo et al., 2019b). The corpus luteum produces the majority of progesterone for the maintenance of pregnancy, but the placentome is known to produce progesterone too (Hoffmann and Schuler, 2002). It is currently unclear as to how this placental progesterone contributes to pregnancy, but alterations in progesterone concentration could feasibly impact the maintenance of pregnancy and abortion. Therefore, placental damage due to infection could also impact progesterone concentrations and lead to abortion in another unexplored pathway.

Future work should include the use of *ex vivo* organ cultures of the bovine placentome and animal studies to truly understand the mechanisms behind bovine abortion. For *ex vivo* organ cultures, bovine placentomes would be collected from cattle slaughtered in commercial abattoirs for human consumption. Evaluation of the approximate pregnancy stage could be conducted using fetal crown-

rump length, as has been carried out by others previously (Miyoshi and Sawamukai, 2004). Placentomes could then be taken back to the laboratory for processing, including treatment with fungicides and antibiotics to reduce the impact of any contaminating microorganisms on the model. Mentioned previously, bacteria expressing GFP or a similar fluorescent marker (validated prior to ensure that expression of GFP does not impact bacterial fitness or virulence) could be flushed through the placental structure to observe bacterial adherence as well as situate the bacteria in the placentomes. After 1h, a subset of samples would be taken for fixation and staining to observe bacteria invading the caruncular cells, based on the work conducted here that 1h is sufficient for a proportion of the bacterial inoculum to invade BCECs.

Work with *ex vivo* organ cultures of the bovine placentome are feasible as others have collected placental material previously for studies into the host response to infection (Silva et al., 2012). However, there are many elements to consider prior to carrying out such work. Most important is the fact that pregnant animals in the third trimester of gestation are prohibited from being transported and slaughtered unless in an emergency (for example, compromised welfare due to illness) by European Union regulations and under RSPCA standards (Off J Eur Union, 2005; RSPCA, 2018). This would restrict the use of pregnant animals in the third trimester unless an animal happened to become unwell and needed to be culled. Additionally, the *ex vivo* environment during culture of placentome explants would be unlikely to mimic the precise hormonal environments which may impact the cellular response to infection. Infiltrating phagocytes would also not be accounted for in this model. Nevertheless, the use of such a model would dramatically improve understanding of the host immune response to infection with *S.* Dublin and the impact this has on the progression of an abortion event.

The study evaluating the immune response in intercotyledonary tissues also demonstrated an increase in TLR4 and TLR5 expression in the final trimester of pregnancy (Silva et al., 2012). This is particularly interesting as many abortions attributed to infection with *S.* Dublin in particular are observed in the final trimester, perhaps indicating increased host responsiveness to infection is implicated in the pathogenesis of *S.* Dublin abortion (Holschbach and Peek, 2018). TLR4 ligands prolong the lifespan of neutrophils for them to exert bactericidal activities, indicating that increased TLR4 expression during the later stages of pregnancy could alter the neutrophil response to infection (Sabroe et al., 2003). Bovine infection with *Neospora caninum* at different times during pregnancy results in different clinical outcomes, attributed in part to the difference in immune response at different gestational time points (Cantón et al., 2014b). Additionally, the impact of infiltrating immune cells on the progression of infection and abortion can only be evaluated in more complex systems. The presence of peripheral blood mononuclear cells is associated with reduced progesterone synthesis from cultured luteal cells (Talbott et al., 2014). This could have an impact on *S.* Dublin-induced abortion as macrophages are recruited to the reproductive tract in response to infection where their presence could reduce progesterone secretion and lead to abortion. These examples highlight the need for animal studies, particularly considering that the impact of the hormonal environment during pregnancy on the maternal immune system is yet to be fully understood, so cannot be accurately modelled *ex vivo*. However, by first elucidating the progression of infection in an *ex vivo* organ culture model of the placentome, the number of animals required for understanding the impact of the hormonal and therefore immune environment would be reduced, in keeping with the Replace, Refine, Reduce guidance from the National Centre for the Replacement, Refinement and Reduction of Animals in Research.

7.5 - Summary

S. Dublin is one of the most common causes of bovine abortion in the UK, presenting a risk to animal health and welfare, as well as being an economic burden to cattle owners and a zoonotic risk threatening human food security. However, relatively little is known about *S.* Dublin and why abortions can occur during infection of pregnant cattle.

S. Dublin isolates from bovine sources in the UK show distinct clustering when phylogenetically aligned based on their accessory genomes. This potentially indicates the presence of host-specific genes conveying pathogenicity in cattle. Clustering seems to be based on genes other than the virulence factors identified in this study, as there was little variation in virulence factors observed in these isolates. Whilst the SPI-2 gene *ssaU* appeared to be noteworthy, its importance in the functionality of the SPI-2 complex and bacterial survival would suggest that the heterologous sequences observed in human-associated isolates may be an artefact of using the VFDB. Nevertheless, further bioinformatic investigation of the *S.* Dublin isolates is warranted, as this would improve understanding of the population of currently circulating isolates in the UK. Furthermore, whilst imperfect, the comparison of *S.* Dublin and *S.* Typhimurium virulence and AMR genes reveals considerable differences between the serovars, demonstrating that serovar-specific research is vital to understanding these pathogens and host specificity individually.

The survival of *S.* Dublin in fresh whole bovine blood suggests that *S.* Dublin may be disseminated in a cell-free niche in the blood and warrants further research into systemic translocation of the bacterium. The experimental data presented in Chapter 4 are consistent with the ability of *S.* Dublin to invade, survive and replicate within BCECs. This demonstrates for the first time the direct infection of placental cells which could lead to cell death, mediated either by the host immune response or by cellular destruction by the bacterium. The production of CXCL8 and TNFα by placental cells in

response to infection with *S.* Dublin is similar to the host response to other, better characterised abortifacient pathogens. This also demonstrates for the first time a subset of the underlying mechanisms of the host response to *S.* Dublin in placental cells which may have detrimental effects to pregnancy. It was not possible to determine any changes in PGE_2 production in response to *S.* Dublin in the experiments conducted in Chapter 5, but it is likely that BCECs would increase the production of PGE_2 as this was observed in response to LPS in a previous study (Collet et al., unpublished observations).

Further research into the genomic factors conveying host specificity, the process of systemic dissemination, and the mechanism behind the host response and abortion is required. Understanding host specificity could lead to the development of vaccines or other management strategies which, without proper characterisation of the bacterium beforehand, would not be possible. Similarly, understanding systemic dissemination of the bacteria and the underlying mechanisms behind abortion could allow for the development of prophylactic measures or treatments to prevent systemic illness caused by *S.* Dublin. This would aid with preventing abortion and could also impact the prevalence of bovine salmonellosis in all age groups more generally.

Chapter 8 - Bibliography

Abby, S. S. and Rocha, E. P. C. (2012). The Non-Flagellar Type III Secretion System Evolved from the Bacterial Flagellum and Diversified into Host-Cell Adapted Systems. *PLoS Genetics*, *8*(9), 1002983. https://doi.org/10.1371/JOURNAL.PGEN.1002983

Achtman, M., Wain, J., Weill, F.-X., Nair, S., Zhou, Z., Sangal, V., Krauland, M. G., Hale, J. L., Harbottle, H., Usebeck, A., Dougan, G., Harrison, L. H. and Brisse, S. (2012). Multilocus Sequence Typing as a Replacement for Serotyping in Salmonella enterica. *PLoS Pathogens*, *8*(6), 1002776. https://doi.org/10.1371/journal.ppat.1002776

Achtman, M., Zhou, Z., Alikhan, N.-F., … Fanning, S. (2020). Genomic diversity of Salmonella enterica -The UoWUCC 10K genomes project. *Wellcome Open Research*, *5*, 223. https://doi.org/10.12688/wellcomeopenres.16291.1

Agard, M., Asakrah, S. and Morici, L. A. (2013). PGE2 suppression of innate immunity during mucosal bacterial infection. *Frontiers in Cellular and Infection Microbiology*, *3*, 45. https://doi.org/10.3389/FCIMB.2013.00045

Agerholm, J. S., Jensen, N. E., Dantzer, V., Jensen, H. E. and Aarestrup, F. M. (1999). Experimental Infection of Pregnant Cows with Bacillus licheniformis Bacteria. *Vet Pathol*, *36*, 191–201.

Ahmad, I., Lamprokostopoulou, A., Le Guyon, S., Streck, E., Barthel, M., Peters, V., Hardt, W. D. and Römling, U. (2011). Complex c-di-GMP Signaling Networks Mediate Transition between Virulence Properties and Biofilm Formation in Salmonella enterica Serovar

Typhimurium. *PLoS ONE*, *6*(12), 28351. https://doi.org/10.1371/JOURNAL.PONE.0028351

Akaogi, J., Yamada, H., Kuroda, Y., Nacionales, D. C., Reeves, W. H. and Satoh, M. (2004). Prostaglandin E2 receptors EP2 and EP4 are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF-alpha and IL-6 production. *Journal of Leukocyte Biology*, *76*(1), 227–236. https://doi.org/10.1189/JLB.1203627

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., … McArthur, A. G. (2020). CARD 2020: Antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Research*, *48*(D1), D517–D525. https://doi.org/10.1093/nar/gkz935

Alikhan, N.-F., Zhou, Z., Sergeant, M. J. and Achtman, M. (2018). A genomic overview of the population structure of Salmonella. *PLOS Genetics*, *14*(4), e1007261. https://doi.org/10.1371/journal.pgen.1007261

Aljahdali, N. H., Sanad, Y. M., Han, J. and Foley, S. L. (2020). Current knowledge and perspectives of potential impacts of Salmonella enterica on the profile of the gut microbiota. *BMC Microbiology*, *20*(1), 353. https://doi.org/10.1186/S12866-020-02008-X

Allert, S., Schulz, D., Kämmer, P., Großmann, P., Wolf, T., Schäuble, S., Panagiotou, G., Brunke, S. and Hube, B. (2022). From environmental adaptation to host survival: Attributes that mediate pathogenicity of Candida auris. *Virulence*, *13*(1), 191. https://doi.org/10.1080/21505594.2022.2026037

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Anderson, M. L. (2007). Infectious causes of bovine abortion during mid- to late-gestation. *Theriogenology*, *68*(3), 474–486. https://doi.org/10.1016/j.theriogenology.2007.04.001

Anderson, R. J., House, J. K., Smith, B. P., Kinde, H., Walker, R. L., Vande Steeg, B. J. and Breitmeyer, R. E. (2001). Epidemiologic and Biological Characteristics of salmonellosis in three dairy herds. *Journal of the American Veterinary Medical Association*, *219*(3), 310–322.

Animal Health Act (1981). The Brucellosis (England) Order 2015, Pub. L. No. 364 (2015). England: The National Archives.

APHA. (2016). *Veterinary Investigation Diagnosis Analysis Monthly Reports from 2002 to 2016*. Retrieved from https://www.gov.uk/government/publications/veterinary-investigation-diagnosis-analysis-vida-report-2014

APHA. (2019a). Abortion Enquiries - APHA Official Veterinarian Instructions. Retrieved February 14, 2019, from http://apha.defra.gov.uk/External_OV_Instructions/Brucellosis/Surveillance/Abortion_Enquiries.html

APHA. (2019b). *Salmonella in Livestock Production in GB 2019*. Retrieved from www.nationalarchives.gov.uk/doc/open-government-licence/version/3/oremailPSI@nationalarchives.gsi.gov.ukwww.gov.uk/apha

APHA. (2021a). APHA Cattle Dashboard - Surveillance Intelligence Unit | Tableau Public. Retrieved November 22, 2021, from https://public.tableau.com/profile/siu.apha#!/vizhome/CattleDashboard/CattleDashboard

APHA. (2021b). Livestock and Wildlife Diseases Diagnosis at APHA - Guidance on sample and test selection.

APHA. (2022). Disease Surveillance Tests. Retrieved May 27, 2022, from https://science.vla.gov.uk/Tests/SearchResults.aspx?SiteName=DST&PriceListCategoryId=3

Asakrah, S., Nieves, W., Mahdi, Z., Agard, M., Zea, A. H., Roy, C. J. and Morici, L. A. (2013). Post-Exposure Therapeutic Efficacy of COX-2 Inhibition against Burkholderia pseudomallei. *PLOS Neglected Tropical Diseases*, *7*(5), e2212. https://doi.org/10.1371/JOURNAL.PNTD.0002212

Auffret, M. D., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., John Wallace, R., Freeman, T. C., Stewart, R., Watson, M. and Roehe, R. (2017). The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. *Microbiome*, *5*(1), 1–11. https://doi.org/10.1186/S40168-017-0378-Z

Avenue, C. (1992). An outbreak of Salmonella dublin infection in England and Wales associated with a soft unpasteurized cows ' milk cheese. *Epidemiology and Infection*, *109*, 389–396.

Baird, G. D., Manning, E. J. and Jones, P. W. (1985). Evidence for related virulence sequences in plasmids of Salmonella dublin and Salmonella typhimurium. *Journal of General Microbiology*, *131*(7), 1815–1823. https://doi.org/10.1099/00221287-131-7-1815

Bajaj, V., Lucas, R. L., Hwang, C. and Lee, C. A. (1996). Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. *Molecular Microbiology*, *22*(4), 703–714. https://doi.org/10.1046/J.1365-2958.1996.D01-1718.X

Baker, S., Griffiths, C. and Nicklin, J. (2011). Measurement of Microbial Growth. In E. Owen (Ed.), *Microbiology, BIOS Instant Notes* (4th ed., pp. 73–74). Oxford: Garland Science.

Ball, P. J. H. and Peters, A. R. (2004a). Anatomy. In *Reproduction in Cattle* (3rd ed., pp. 13–27).

Ball, P. J. H. and Peters, A. R. (2004b). Reproductive Efficiency in Cattle Production. In *Reproduction in Cattle* (3rd ed., pp. 1–12).

Baranova, N. and Nikaido, H. (2002). The BaeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate. *Journal of Bacteriology*, *184*(15), 4168–4176. https://doi.org/10.1128/JB.184.15.4168-4176.2002

Barber, E. M., Fazzari, M. and Pollard, J. W. (2005). Th1 cytokines are essential for placental immunity to Listeria monocytogenes. *Infection and Immunity*, *73*(10), 6322–6331. https://doi.org/10.1128/IAI.73.10.6322-6331.2005

Barkallah, M., Gharbi, Y., Hassena, A. Ben, Slima, A. Ben, Mallek, Z., Gautier, M., Greub, G., Gdoura, R. and Fendri, I. (2014). Survey of Infectious Etiologies of Bovine Abortion during Mid- to Late Gestation in Dairy Herds. *PLoS ONE*, *9*(3), e91549. https://doi.org/10.1371/journal.pone.0091549

Barnhart, M. M. and Chapman, M. R. (2006). Curli Biogenesis and Function. *Annual Review of Microbiology*, *60*, 131–147. https://doi.org/10.1146/annurev.micro.60.080805.142106

Basnyat, B., Qamar, F. N., Rupali, P., Ahmed, T. and Parry, C. M. (2021). Clinical update: Enteric fever. *BMJ*, *372*, n437. https://doi.org/10.1136/BMJ.N437

Bäumler, A. J., Tsolis, R. M., Bowe, F. A., Kusters, J. G., Hoffmann, S. and Heffron, F. (1996). The pef fimbrial operon of Salmonella typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. *Infection*

and Immunity, *64*(1), 61–68. https://doi.org/10.1128/IAI.64.1.61-68.1996

Baumler, A. J., Tsolis, R. M., Ficht, T. A. and Adams, L. G. (1998). Evolution of Host Adaptation in Salmonella enterica. *INFECTION AND IMMUNITY*, *66*(10), 4579–4587.

Bäumler, A. J., Tsolis, R. M. and Heffron, F. (1996). The lpf fimbrial operon mediates adhesion of Salmonella typhimurium to murine Peyer's patches. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(1), 279–283. https://doi.org/10.1073/PNAS.93.1.279

Baxter, M. A. and Jones, B. D. (2005). The fimYZ Genes Regulate Salmonella enterica Serovar Typhimurium Invasion in Addition to Type 1 Fimbrial Expression and Bacterial Motility. *Infection and Immunity*, *73*(3), 1377. https://doi.org/10.1128/IAI.73.3.1377-1385.2005

Becker, J., Tchagou Tchangou, G. E., Schmidt, S., Zelent, C., Kahl, F. and Wilting, J. (2020). Absence of lymphatic vessels in term placenta. *BMC Pregnancy and Childbirth*, *20*(1), 1–8. https://doi.org/10.1186/S12884-020-03073-W

Benz, J., Reinstein, J. and Meinhart, A. (2013). Structural Insights into the Effector - Immunity System Tae4/Tai4 from Salmonella typhimurium. *PloS One*, *8*(6), e67362. https://doi.org/10.1371/JOURNAL.PONE.0067362

Berg, H. C. (2003). The Rotary Motor of Bacterial Flagella. *Annual Review of Biochemistry*, *72*, 19–54. https://doi.org/10.1146/ANNUREV.BIOCHEM.72.121801.161737

Beshiru, A., Igbinosa, I. H. and Igbinosa, E. O. (2018). Biofilm formation and potential virulence factors of Salmonella strains

isolated from ready-to-eat shrimps. *PLoS ONE*, *13*(9), e0204345. https://doi.org/10.1371/JOURNAL.PONE.0204345

Betancor, L., Yim, L., Martínez, A., Fookes, M., Sasias, S., Schelotto, F., Thomson, N., Maskell, D. and Chabalgoity, J. A. (2012). Genomic Comparison of the Closely Related Salmonella enterica Serovars Enteritidis and Dublin. *The Open Microbiology Journal*, *6*, 5–13. https://doi.org/10.2174/1874285801206010005

Betancourt, D. M., Llana, M. N., Sarnacki, S. H., Cerquetti, M. C., Monzalve, L. S., Pustovrh, M. C. and Giacomodonato, M. N. (2021). Salmonella Enteritidis foodborne infection induces altered placental morphometrics in the murine model. *Placenta*, *109*, 11–18. https://doi.org/10.1016/J.PLACENTA.2021.04.004

Betbeze, C. and McLaughlin, R. (2002). Canine diskospondylitis: Its etiology, diagnosis, and treatment. *Veterinary Medicine*, *97*(9), 673–681.

Betz, M. and Fox, B. S. (1991). Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *The Journal of Immunology*, *146*(1), 108–113.

Beuzón, C. R., Méresse, S., Unsworth, K. E., Ruíz-Albert, J., Garvis, S., Waterman, S. R., Ryder, T. A., Boucrot, E. and Holden, D. W. (2000). Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. *The EMBO Journal*, *19*(13), 3235–3249. https://doi.org/10.1093/EMBOJ/19.13.3235

Bicalho, M. L. S., Lima, F. S., Machado, V. S., Meira, E. B., Ganda, E. K., Foditsch, C., Bicalho, R. C. and Gilbert, R. O. (2016). Associations among Trueperella pyogenes, endometritis diagnosis, and pregnancy outcomes in dairy cows. *Theriogenology*, *85*(2), 267–274. https://doi.org/10.1016/j.theriogenology.2015.09.043

Blanchard, A. M., Billenness, R., Warren, J., Glanvill, A., Roden, W., Drinkall, E., Maboni, G., Robinson, R. S., Rees, C. E. D., Pfarrer, C. and Tötemeyer, S. (2019). Sensitivity of Listeria monocytogenes to lysozyme predicts ability to proliferate in bovine caruncular epithelial cells. *BioRxiv*, 855841. https://doi.org/10.1101/855841

Blanchard, A. M., Billenness, R., Warren, J., Glanvill, A., Roden, W., Drinkall, E., Maboni, G., Robinson, R. S., Rees, C. E. D., Pfarrer, C. and Tötemeyer, S. (2020). Characterisation of Listeria monocytogenes isolates from cattle using a bovine caruncular epithelial cell model. *Heliyon*, *6*(7), e04476. https://doi.org/10.1016/j.heliyon.2020.e04476

Bleasdale, B., Lott, P. J., Jagannathan, A., Stevens, M. P., Birtles, R. J. and Wigley, P. (2009). The Salmonella Pathogenicity Island 2-Encoded Type III Secretion System Is Essential for the Survival of Salmonella enterica Serovar Typhimurium in Free-Living Amoebae. *Applied and Environmental Microbiology*, *75*(6), 1793–1795. https://doi.org/10.1128/AEM.02033-08

Blondel, C. J., Jiménez, J. C., Contreras, I. and Santiviago, C. A. (2009). Comparative genomic analysis uncovers 3 novel loci encoding type six secretion systems differentially distributed in Salmonella serotypes. *BMC Genomics*, *10*(1), 1–17. https://doi.org/10.1186/1471-2164-10-354

Blondel, C. J., Jiménez, J. C., Leiva, L. E., Álvarez, S. A., Pinto, B. I., Contreras, F., Pezoa, D., Santiviago, C. A. and Contreras, I. (2013). The type VI secretion system encoded in salmonella pathogenicity island 19 is required for Salmonella enterica serotype gallinarum survival within infected macrophages. *Infection and Immunity*, *81*(4), 1207–1220. https://doi.org/10.1128/IAI.01165-12

Blondel, C. J., Yang, H. J., Castro, B., Chiang, S., Toro, C. S., Zaldïvar, M., Contreras, I., Andrews-Polymenis, H. L. and Santiviago, C. A.

(2010). Contribution of the Type VI Secretion System Encoded in SPI-19 to Chicken Colonization by Salmonella enterica Serotypes Gallinarum and Enteritidis. *PLOS ONE*, *5*(7), e11724. https://doi.org/10.1371/JOURNAL.PONE.0011724

Boddicker, J. D., Ledeboer, N. A., Jagnow, J., Jones, B. D. and Clegg, S. (2002). Differential binding to and biofilm formation on, HEp-2 cells by Salmonella enterica Serovar Typhimurium is dependent upon allelic variation in the fimH gene of the fim gene cluster. *Molecular Microbiology*, *45*(5), 1255–1265. https://doi.org/10.1046/J.1365-2958.2002.03121.X

Bolton, A. J., Osborne, M. P., Wallis, T. S. and Stephen, J. (1999). Interaction of Salmonella choleraesuis, Salmonella dublin and Salmonella typhimurium with porcine and bovine terminal ileum in vivo. *Microbiology*, *145*, 2431–2441. https://doi.org/10.1099/00221287-145-9-2431

Bondurant, R. H. (1999). Inflammation in the bovine female reproductive tract. *Journal of Animal Science*, *77*, 101–110. https://doi.org/10.2527/1999.77suppl_2101x

Bonifield, H. R. and Hughes, K. T. (2003). Flagellar Phase Variation in Salmonella enterica Is Mediated by a Posttranscriptional Control Mechanism. *Journal of Bacteriology*, *185*(12), 3567. https://doi.org/10.1128/JB.185.12.3567-3574.2003

Borges, Á. M., Healey, G. D. and Sheldon, I. M. (2012). Explants of Intact Endometrium to Model Bovine Innate Immunity and Inflammation Ex Vivo. *American Journal of Reproductive Immunology*, *67*(6), 526–539. https://doi.org/10.1111/J.1600-0897.2012.01106.X

Bougarn, S., Cunha, P., Gilbert, F., Meurens, F. and Rainard, P. (2011). Technical note: Validation of candidate reference genes for normalization of quantitative PCR in bovine mammary epithelial cells responding to inflammatory stimuli. *Journal of*

Dairy Science, *94*, 2425–2430. https://doi.org/10.3168/jds.2010-3859

Bowman, C. C. and Bost, K. L. (2009). Cyclooxygenase-2 Inhibition Enhances Activation of T Helper Type 1 Responses During Salmonella Infection. *The Open Microbiology Journal*, *3*(1), 23. https://doi.org/10.2174/1874285800903010023

Braibant, M., Chevalier, J., Chaslus-Dancla, E., Pagès, J. M. and Cloeckaert, A. (2005). Structural and Functional Study of the Phenicol-Specific Efflux Pump FloR Belonging to the Major Facilitator Superfamily. *Antimicrobial Agents and Chemotherapy*, *49*(7), 2965. https://doi.org/10.1128/AAC.49.7.2965-2971.2005

Brambell, F. W. R., Barbour, D. S., Barnett, M. B., Ewer, T. K., Hobson, A., Pitchforth, H., Smith, W. R., Thorpe, W. H. and Winship, F. J. W. (1965). *Report to the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems*. London.

Braukmann, M., Methner, U. and Berndt, A. (2015). Avian CD25+ gamma/delta (γδ) T cells after Salmonella exposure. *Veterinary Immunology and Immunopathology*, *168*(1–2), 14–18. https://doi.org/10.1016/J.VETIMM.2015.09.010

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R. and Swaminathan, B. (2000). Salmonella Nomenclature. *Journal of Clinical Microbiology*, *38*(7), 2465. https://doi.org/10.1128/jcm.38.7.2465-2467.2000

Bridger, P. S., Haupt, S., Klisch, K., Leiser, R., Tinneberg, H. and Pfarrer, C. (2007). Validation of primary epitheloid cell cultures isolated from bovine placental caruncles and cotyledons. *Theriogenology*, *68*(4), 592–603. https://doi.org/10.1016/j.theriogenology.2007.05.046

Bridger, P. S., Menge, C., Leiser, R., Tinneberg, H. R. and Pfarrer, C. D. (2007). Bovine Caruncular Epithelial Cell Line (BCEC-1) Isolated from the Placenta Forms a Functional Epithelial Barrier in a Polarised Cell Culture Model. *Placenta*, *28*(11–12), 1110–1117. https://doi.org/10.1016/j.placenta.2007.07.002

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. and Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science*, *303*(5663), 1532–1535. https://doi.org/10.1126/SCIENCE.1092385

Bronner, A., Hénaux, V., Fortané, N., Hendrikx, P. and Calavas, D. (2014). Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? *BMC Veterinary Research*, *10*(1), 1–12. https://doi.org/10.1186/1746-6148-10-93

Broz, P., Ohlson, M. B. and Monack, D. M. (2012). Innate immune response to Salmonella typhimurium, a model enteric pathogen. *Gut Microbes*, *3*(2), 62. https://doi.org/10.4161/GMIC.19141

Buchmeiert, N. A. and Heffront, F. (1991). Inhibition of Macrophage Phagosome-Lysosome Fusion by Salmonella typhimurium. *INFECTION AND IMMUNITY*, *59*(7), 2232–2238.

Budras, K. D., Habel, Robert. E., Mulling, C. K. W., Greenough, P. R., Jahrmarker, G., Richter, R. and Starke, D. (2011). *Bovine Anatomy: An Illustrated Text* (2nd ed.). Schluetersche.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. and Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Cabell, E. (2007). Bovine abortion: Aetiology and investigations. *In Practice*, *29*(8), 455–463. https://doi.org/10.1136/inpract.29.8.455

Cahilog, Z., Zhao, H., Wu, L., Alam, A., Eguchi, S., Weng, H. and Ma, D. (2020). The Role of Neutrophil NETosis in Organ Injury: Novel Inflammatory Cell Death Mechanisms. *Inflammation*, *43*(6), 2021. https://doi.org/10.1007/S10753-020-01294-X

Cain, R. J., Hayward, R. D. and Koronakis, V. (2008). Deciphering interplay between Salmonella invasion effectors. *PLoS Pathogens*, *4*(4), e1000037. https://doi.org/10.1371/journal.ppat.1000037

Cantón, G. J., Katzer, F., Maley, S. W., Bartley, P. M., Benavides-Silván, J., Palarea-Albaladejo, J., Pang, Y., Smith, S. H., Rocchi, M., Buxton, D., Innes, E. A. and Chianini, F. (2014a). Cytokine expression in the placenta of pregnant cattle after inoculation with Neospora caninum. *Veterinary Immunology and Immunopathology*, *161*(1–2), 77–89. https://doi.org/10.1016/J.VETIMM.2014.07.004

Cantón, G. J., Katzer, F., Maley, S. W., Bartley, P. M., Benavides-Silván, J., Palarea-Albaladejo, J., Pang, Y., Smith, S. H., Rocchi, M. S., Buxton, D., Innes, E. A. and Chianini, F. (2014b). Inflammatory infiltration into placentas of Neospora caninum challenged cattle correlates with clinical outcome of pregnancy. *Veterinary Research*, *45*(1), 1–6. https://doi.org/10.1186/1297-9716-45-11

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., Aarestrup, F. M. and Hasman, H. (2014). In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3895. https://doi.org/10.1128/AAC.02412-14

Carpenter, T. E., Chrièl, M., Andersen, M. M., Wulfson, L., Jensen, A. M., Houe, H. and Greiner, M. (2006). An epidemiologic study of late-term abortions in dairy cattle in Denmark, July 2000-August 2003. *Preventive Veterinary Medicine*, *77*(3–4), 215–229. https://doi.org/10.1016/j.prevetmed.2006.07.005

Carrique-Mas, J. J., Willmington, J. A., Papadopoulou, C., Watson, E. N. and Davies, R. H. (2010). Salmonella infection in cattle in Great Britain, 2003 to 2008. *The Veterinary Record*, *167*(15), 560–565. https://doi.org/10.1136/vr.c4943

Carvalho Neta, A. V., Stynen, A. P. R., Paixão, T. A., Miranda, K. L., Silva, F. L., Roux, C. M., Tsolis, R. M., Everts, R. E., Lewin, H. A., Adams, L. G., Carvalho, A. F., Lage, A. P. and Santos, R. L. (2008). Modulation of the Bovine Trophoblastic Innate Immune Response by Brucella abortus. *Infection and Immunity*, *76*(5), 1897. https://doi.org/10.1128/IAI.01554-07

Castro, E., Tony Parks, W. and Galambos, C. (2011). Neither normal nor diseased placentas contain lymphatic vessels. *Placenta*, *32*(4), 310–316. https://doi.org/10.1016/J.PLACENTA.2011.01.013

Cerminati, S., Giri, G. F., Mendoza, J. I., Soncini, F. C. and Checa, S. K. (2017). The CpxR/CpxA system contributes to Salmonella gold-resistance by controlling the GolS-dependent gesABC transcription. *Environmental Microbiology*, *19*(10), 4035–4044. https://doi.org/10.1111/1462-2920.13837

Cervantes-Barragán, L., Gil-Cruz, C., Pastelin-Palacios, R., Lang, K. S., Isibasi, A., Ludewig, B. and López-Macías, C. (2009). TLR2 and TLR4 signaling shapes specific antibody responses to Salmonella typhi antigens. *European Journal of Immunology*, *39*(1), 126–135. https://doi.org/10.1002/EJI.200838185

Chanrot, M., Blomqvist, G., Guo, Y., Ullman, K., Juremalm, M., Bage, R., Donofrio, G., Valarcher, J. F. and Humblot, P. (2017). Bovine

herpes virus type 4 alters TNF-α and IL-8 profiles and impairs the survival of bovine endometrial epithelial cells. *Reproductive Biology*, *17*(3), 225–232. https://doi.org/10.1016/J.REPBIO.2017.05.006

Chaouat, G., Menu, E., Clark, D. A., Dy, M., Minkowski, M. and Wegmann, T. G. (1990). Control of fetal survival in CBA × DBA/2 mice by lymphokine therapy. *Reproduction*, *89*(2), 447–458. https://doi.org/10.1530/JRF.0.0890447

Chen, H. M., Wang, Y., Su, L. H. and Chiu, C. H. (2013). Nontyphoid Salmonella infection: Microbiology, clinical features, and antimicrobial therapy. *Pediatrics and Neonatology*, *54*(3), 147–152. https://doi.org/10.1016/j.pedneo.2013.01.010

Cheraghchi, N., Khaki, P., Bidhendi, S. M. and Sabokbar, A. (2014). Identification of Isolated Salmonella enterica Serotype gallinarum Biotype Pullorum and Gallinarum by PCR-RFLP. *Jundishapur Journal of Microbiology*, *7*(9), 19135. https://doi.org/10.5812/JJM.19135

Chu, C. H., Feng, Y., Chien, A. C., Hu, S., Chu, C. H. and Chiu, C. H. (2008). Evolution of genes on the Salmonella Virulence plasmid phylogeny revealed from sequencing of the virulence plasmids of S. enterica serotype Dublin and comparative analysis. *Genomics*, *92*(5), 339–343. https://doi.org/10.1016/j.ygeno.2008.07.010

Cianfanelli, F. R., Monlezun, L. and Coulthurst, S. J. (2016). Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon. *Trends in Microbiology*, *24*(1), 51–62. https://doi.org/10.1016/J.TIM.2015.10.005

Clardy, J., Fischbach, M. A. and Currie, C. R. (2009). The natural history of antibiotics. *Current Biology*, *19*(11), 437–441. https://doi.org/10.1016/J.CUB.2009.04.001

Collet, H., Schäfer, I., Wood, E., Pfarrer, C., Wapenaar, W. and Totemeyer, S. (n.d.). Innate immune response elicited by host pathogen interactions of bovine caruncular cells with abortifacient bacteria Leptospira borgpetersenii serotype Hardjo Bovis and Listeria monocytogenes. *Unpublished*.

Collier-Hyams, L. S., Zeng, H., Sun, J., Tomlinson, A. D., Bao, Z. Q., Chen, H., Madara, J. L., Orth, K. and Neish, A. S. (2002). Salmonella AvrA Effector Inhibits the Key Proinflammatory, Anti-Apoptotic NF-κB Pathway. *The Journal of Immunology*, *169*(6), 2846–2850. https://doi.org/10.4049/jimmunol.169.6.2846

Committee on Reproductive Nomenclature Recommendations for Standardising Bovine Reproductive Terms. (1972). *The Cornell Veterinarian*, *62*(2), 216–237.

Conroy, O., Kim, E. H., McEvoy, M. M. and Rensing, C. (2010). Differing ability to transport non-metal substrates by two RND-type metal exporters. *FEMS Microbiology Letters*, *308*(2), 115. https://doi.org/10.1111/J.1574-6968.2010.02006.X

Cossart, P. (2004). Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens. *Science*, *304*(5668), 242–248. https://doi.org/10.1126/science.1090124

Coutinho, L. B., Gomes, A. O., Araújo, E. C. B., Barenco, P. V. C., Santos, J. L., Caixeta, D. R., Silva, D. A. O., Cunha-Júnior, J. P., Ferro, E. A. V. and Silva, N. M. (2012). The impaired pregnancy outcome in murine congenital toxoplasmosis is associated with a pro-inflammatory immune response, but not correlated with decidual inducible nitric oxide synthase expression. *International Journal for Parasitology*, *42*(4), 341–352. https://doi.org/10.1016/J.IJPARA.2012.01.006

Cox, C. E., Wright, A. C., McClelland, M. and Teplitski, M. (2016). Influence of Salmonella enterica serovar typhimurium ssrB on colonization of eastern oysters (Crassostrea virginica) as

revealed by a promoter probe screen. *Applied and Environmental Microbiology*, *82*(1), 328–339. https://doi.org/10.1128/AEM.02870-15

Cresswell, E., Brennan, M. L., Barkema, H. W. and Wapenaar, W. (2014). A questionnaire-based survey on the uptake and use of cattle vaccines in the UK. *VetRecord Open*, *1*(1), e000042. https://doi.org/10.1136/vropen-2014

Cronin, J. G., Turner, M. L., Goetze, L., Bryant, C. E. and Sheldon, I. M. (2012). Toll-like Receptor 4 and MyD88 Dependent Signaling Mechanisms of the Innate Immune System are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium. *Biology of Reproduction*, *86*(2), 51. https://doi.org/10.1095/BIOLREPROD.111.092718

Crump, J. A., Sjölund-Karlsson, M., Gordon, M. A. and Parry, C. M. (2015). Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. *Clinical Microbiology Reviews*, *28*(4), 901. https://doi.org/10.1128/CMR.00002-15

Cui, L., Wang, X., Zhao, Y., Peng, Z., Gao, P., Cao, Z., Feng, J., Zhang, F., Guo, K., Wu, M., Chen, H. and Dai, M. (2021). Virulence comparison of salmonella enterica subsp. Enterica isolates from chicken and whole genome analysis of the high virulent strain s. enteritidis 211. *Microorganisms*, *9*(11), 2239. https://doi.org/10.3390/MICROORGANISMS9112239

Cummings, K. J., Rodriguez-Rivera, L. D., Capel, M. B., Rankin, S. C. and Nydam, D. V. (2019). Short communication: Oral and intranasal administration of a modified-live Salmonella Dublin vaccine in dairy calves: Clinical efficacy and serologic response. *Journal of Dairy Science*, *102*(4), 3474–3479. https://doi.org/10.3168/jds.2018-14892

Cutler, S. J., Whatmore, A. M. and Commander, N. J. (2004). Brucellosis-new aspects of an old disease. *Journal of Applied Microbiology*, *98*, 1270–1281. https://doi.org/10.1111/j.1365-2672.2005.02622.x

da Silva, C. V., Cruz, L., Araújo, N. da S., Angeloni, M. B., Fonseca, B. B., Gomes, A. de O., Carvalho, F. dos R., Gonçalves, A. L. R. and Barbosa, B. de F. (2012). A glance at Listeria and Salmonella cell invasion: Different strategies to promote host actin polymerization. *International Journal of Medical Microbiology*, *302*(1), 19–32. https://doi.org/10.1016/j.ijmm.2011.05.003

Darboe, S., Bradbury, R., Phelan, J., Kanteh, A., Muhammad, A.-K., Worwui, A., Yang, S., Nwakanma, D., Perez-Sepulveda, B., Kariuki, S., Kwambana-Adams, B. and Antonio, M. (2020). Genomic diversity and antimicrobial resistance among non-typhoidal Salmonella associated with human disease in The Gambia. *PREPRINT*. Retrieved from https://www.biorxiv.org/content/10.1101/2020.10.30.316588v1.full.pdf

Darboe, S., Bradbury, R. S., Phelan, J., Kanteh, A., Muhammad, A. K., Worwui, A., Yang, S., Nwakanma, D., Perez-Sepulveda, B., Kariuki, S., Kwambana-Adams, B. and Antonio, M. (2022). Genomic diversity and antimicrobial resistance among non-typhoidal Salmonella associated with human disease in The Gambia. *Microbial Genomics*, *8*(3), 785. https://doi.org/10.1099/MGEN.0.000785

Darwin, K. H. and Miller, V. L. (1999). Molecular basis of the interaction of Salmonella with the intestinal mucosa. *Clinical Microbiology Reviews*, *12*(3), 405–428. https://doi.org/10.1128/cmr.12.3.405

Davis, M. A., Besser, T. E., Orfe, L. H., Baker, K. N. K., Lanier, A. S., Broschat, S. L., New, D. and Call, D. R. (2011). Genotypic-Phenotypic Discrepancies between Antibiotic Resistance

Characteristics of Escherichia coli Isolates from Calves in Management Settings with High and Low Antibiotic Use. *Applied and Environmental Microbiology*, *77*(10), 3293. https://doi.org/10.1128/AEM.02588-10

Davison, H. C., Sayers, A. R., Smith, R. P., Pascoe, S. J. S., Davies, R. H., Weaver, J. P. and Evans, S. J. (2006). Risk factors associated with the salmonella status of dairy farms in England and Wales. *Veterinary Record*, *159*, 871–880. https://doi.org/10.1136/vr.159.26.871

Davison, H. C., Smith, R. P., Pascoe, S. J. S., Sayers, A. R., Davies, R. H., Weaver, J. P., Kidd, S. A., Dalziel, R. W. and Evans, S. J. (2005). Prevalence, incidence and geographical distribution of serovars of Salmonella on dairy farms in England and Wales. *Veterinary Record*, *157*(22), 703–711. https://doi.org/10.1136/vr.157.22.703

D'Costa, V. M., Braun, V., Landekic, M., Shi, R., Proteau, A., McDonald, L., Cygler, M., Grinstein, S. and Brumell, J. H. (2015). Salmonella Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7. *Cell Reports*, *12*(9), 1508–1518. https://doi.org/10.1016/J.CELREP.2015.07.063

de Oliveira, S., Lopez-Muñoz, A., Martínez-Navarro, F. J., Galindo-Villegas, J., Mulero, V. and Calado, Â. (2015). Cxcl8-l1 and Cxcl8-l2 are required in the zebrafish defense against Salmonella Typhimurium. *Developmental & Comparative Immunology*, *49*(1), 44–48. https://doi.org/10.1016/J.DCI.2014.11.004

Dealtry, G., O-Farrell, M. and Fernandez, N. (2000). The Th2 Cytokine Environment of the Placenta. *International Archives of Allergy and Immunology*, *123*(2), 107–119. https://doi.org/10.1159/000024441

Deen, J., von Seidlein, L., Andersen, F., Elle, N., White, N. J. and Lubell, Y. (2012). Community-acquired bacterial bloodstream

infections in developing countries in south and southeast Asia: a systematic review. *The Lancet Infectious Diseases*, *12*(6), 480–487. https://doi.org/10.1016/S1473-3099(12)70028-2

DEFRA. (2004). *Brucellosis A reminder for all Cattle farmers*.

DEFRA. (2018). Official Veterinarian Instructions. Retrieved March 12, 2019, from http://apha.defra.gov.uk/External_OV_Instructions/Brucellosis/Surveillance/Abortion_Enquiries.html

Delwick, J., Nikolaus, T., Erdogan, S. and Hensel, M. (1999). Environmental regulation of Salmonella pathogenicity island 2 gene expression. *Molecular Microbiology*, *31*(6), 1759–1773. https://doi.org/10.1046/J.1365-2958.1999.01312.X

Deng, W., Marshall, N. C., Rowland, J. L., McCoy, J. M., Worrall, L. J., Santos, A. S., Strynadka, N. C. J. and Brett Finlay, B. (2017). Assembly, structure, function and regulation of type III secretion systems. *Nature Reviews Microbiology*, *15*, 323–337. https://doi.org/10.1038/nrmicro.2017.20

Dias, M., Antony, B., Pinto, H. and Rekha, B. (2009). Salmonella enterica serotype Dublin bacteraemia mimicking enteric fever. *Indian Journal of Medical Microbiology*, *27*(4), 365–367. https://doi.org/10.4103/0255-0857.55463

Dietsche, T., Tesfazgi, M. M., Brunner, M. J., Abrusci, P., Yan, J., Franz-Wachtel, M., Schä Rfe, C., Zilkenat, S., Grin, I., Galá, J. E., Kohlbacher, O., Lea, S., Macek, B., Marlovits, T. C., Robinson, C. V and Wagner, S. (2016). Structural and Functional Characterization of the Bacterial Type III Secretion Export Apparatus. *PLOS Pathogens*, *12*(12), e1006071. https://doi.org/10.1371/journal.ppat.1006071

dos Santos, A. M. P., Ferrari, R. G., Panzenhagen, P., Rodrigues, G. L. and Conte-Junior, C. A. (2021). Virulence genes identification

and characterization revealed the presence of the Yersinia High Pathogenicity Island (HPI) in Salmonella from Brazil. *Gene*, *787*, 145646. https://doi.org/10.1016/J.GENE.2021.145646

Dostal, A., Gagnon, M., Chassard, C., Zimmermann, M. B., O'mahony, L. and Lacroix, C. (2014). Salmonella Adhesion, Invasion and Cellular Immune Responses Are Differentially Affected by Iron Concentrations in a Combined In Vitro Gut Fermentation-Cell Model. *PLoS ONE*, *9*(3), 93549. https://doi.org/10.1371/journal.pone.0093549

Doster, E., Lakin, S. M., Dean, C. J., Wolfe, C., Young, J. G., Boucher, C., Belk, K. E., Noyes, N. R. and Morley, P. S. (2020). MEGARes 2.0: A database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Nucleic Acids Research*, *48*(D1), D561–D569. https://doi.org/10.1093/nar/gkz1010

Doublet, B., Boyd, D., Mulvey, M. R. and Cloeckaert, A. (2005). The Salmonella genomic island 1 is an integrative mobilizable element. *Molecular Microbiology*, *55*(6), 1911–1924. https://doi.org/10.1111/j.1365-2958.2005.04520.x

Echenique-Rivera, H., Muzzi, A., Del Tordello, E., Seib, K. L., Francois, P., Rappuoli, R., Pizza, M. and Serruto, D. (2011). Transcriptome Analysis of Neisseria meningitidis in Human Whole Blood and Mutagenesis Studies Identify Virulence Factors Involved in Blood Survival. *PLoS Pathogens*, *7*(5), 1002027. https://doi.org/10.1371/JOURNAL.PPAT.1002027

Eckmann, L., Kagnoff, M. F. and Fierer, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infection and Immunity*, *61*(11), 4569. https://doi.org/10.1128/iai.61.11.4569-4574.1993

El Sayed, F., Sapriel, G., Fawal, N., Gruber, A., Bauer, T., Heym, B., Dupont, C., Garchon, H.-J., Gaillard, J.-L., Rottman, M. and Le

Hello, S. (2018). In-Host Adaptation of Salmonella enterica Serotype Dublin during Prosthetic Hip Joint Infection. *Emerging Infectious Diseases*, *24*(12), 2360–2363. https://doi.org/10.3201/eid2412.180214

Elsinghorst, E. A. (1994). Measurement of invasion by gentamicin resistance. *Methods in Enzymology*, *236*, 405–420. https://doi.org/10.1016/0076-6879(94)36030-8

Entrican, G. (2002). Immune Regulation during Pregnancy and Host-Pathogen Interactions in Infectious Abortion. *J. Comp. Path*, *126*(2–3), 79–94. https://doi.org/10.1053/jcpa.2001.0539

Entrican, G., Buxton, D. and Longbottom, D. (2001). Chlamydial infection in sheep: immune control versus fetal pathology. *Journal of the Royal Society of Medicine*, *94*(6), 273. https://doi.org/10.1177/014107680109400605

Ersbøll, A. K. and Nielsen, L. R. (2008). The range of influence between cattle herds is of importance for the local spread of Salmonella Dublin in Denmark. *Preventive Veterinary Medicine*, *84*(3–4), 277–290. https://doi.org/10.1016/j.prevetmed.2007.12.005

Fair, T. (2015). The Contribution of the Maternal Immune System to the Establishment of Pregnancy in Cattle. *Frontiers in Immunology*, *6*(7). https://doi.org/10.3389/FIMMU.2015.00007

Fang, F. C. and Fierer, J. (1991). Human infection with Salmonella Dublin. *Medicine*, *70*(3), 198–207. https://doi.org/10.1097/00005792-199105000-00004

Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S. and Gordon, M. A. (2012). Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *The Lancet*, *379*(9835), 2489–2499. https://doi.org/10.1016/S0140-6736(11)61752-2

Feng, Y., Liu, J., Li, Y. G., Cao, F. L., Johnston, R. N., Zhou, J., Liu, G. R. and Liu, S. L. (2012). Inheritance of the Salmonella virulence plasmids: Mostly vertical and rarely horizontal. *Infection, Genetics and Evolution*, *12*(5), 1058–1063. https://doi.org/10.1016/J.MEEGID.2012.03.004

Fenton, S. E., Clough, H. E., Diggle, P. J., Evans, S. J., Davison, H. C., Vink, W. D. and French, N. P. (2009). Spatial and spatio-temporal analysis of Salmonella infection in dairy herds in England and Wales. *Epidemiology and Infection*, *137*(6), 847–857. https://doi.org/10.1017/S0950268808001349

Feria, J. V. M., Lefebre, M. D., Stierhof, Y.-D., Galán, J. E., Wagner, S. and Kolter, R. (2015). Role of Autocleavage in the Function of a Type III Secretion Specificity Switch Protein in Salmonella enterica Serovar Typhimurium. *MBio*, *13*(6), e01459. https://doi.org/10.1128/mBio.01459-15

Fernandes, M. M., Ivanova, K., Hoyo, J., Pérezpérez-Rafael, S., Francesko, A. and Tzanov, T. (2017). Nanotransformation of Vancomycin Overcomes the Intrinsic Resistance of Gram-Negative Bacteria. *ACS Applied Materials and Interfaces*, *9*(17), 15022–15030. https://doi.org/10.1021/acsami.7b00217

Figueira, R., Watson, K. G., Holden, D. W. and Helaine, S. (2013). Identification of salmonella pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of S. enterica serovar typhimurium: implications for rational vaccine design. *MBio*, *4*(2), e00065. https://doi.org/10.1128/MBIO.00065-13

Figueroa-Bossi, N. and Bossi, L. (1999). Inducible prophages contribute to Salmonella virulence in mice. *Molecular Microbiology*, *33*(1), 167–176. https://doi.org/10.1046/J.1365-2958.1999.01461.X

Folkesson, A., Löfdahl, S. and Normark, S. (2002). The Salmonella enterica subspecies I specific centisome 7 genomic island encodes novel protein families present in bacteria living in close contact with eukaryotic cells. *Research in Microbiology*, *153*(8), 537–545. https://doi.org/10.1016/S0923-2508(02)01348-7

Frankel, G., Newton, S. M. C., Schoolnik, G. K. and Stocker, B. A. D. (1989). Intragenic recombination in a flagellin gene: characterization of the H1-j gene of Salmonella typhi. *EMBO Journal*, *8*(10), 3149–3152. https://doi.org/10.1002/J.1460-2075.1989.TB08468.X

Frost, A. J., Bland, A. P. and Wallis, T. S. (1997). The Early Dynamic Response of the Calf Ileal Epithelium to Salmonella typhimurium. *Veterinary Pathology*, *34*(5), 369–386. https://doi.org/10.1177/030098589703400501

Frye, J., Karlinsey, J. E., Felise, H. R., Marzolf, B., Dowidar, N., McClelland, M. and Hughes, K. T. (2006). Identification of New Flagellar Genes of Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology*, *188*(6), 2233. https://doi.org/10.1128/JB.188.6.2233-2243.2006

Fujino, H., Salvi, S. and Regan, J. W. (2005). Differential Regulation of Phosphorylation of the cAMP Response Element-Binding Protein after Activation of EP2 and EP4 Prostanoid Receptors by Prostaglandin E2. *Molecular Pharmacology*, *68*(1), 251–259. https://doi.org/10.1124/MOL.105.011833

García-Soto, S., Tomaso, H., Linde, J. and Methner, U. (2021). Epidemiological Analysis of Salmonella enterica subsp. enterica Serovar Dublin in German Cattle Herds Using Whole-Genome Sequencing . *Microbiology Spectrum*, *9*(2). https://doi.org/10.1128/SPECTRUM.00332-21

Giaglis, S., Stoikou, M., Chowdhury, C. S., Schaefer, G., Grimolizzi, F., Rossi, S. W., Hoesli, I. M., Lapaire, O., Hasler, P. and Hahn,

S. (2016). Multimodal regulation of NET formation in pregnancy: Progesterone antagonizes the pro-NETotic effect of estrogen and G-CSF. *Frontiers in Immunology*, *7*, 1. https://doi.org/10.3389/FIMMU.2016.00565

Giannella, R. A., Formal, S. B., Dammin, G. J. and Collins, H. (1973). Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. *The Journal of Clinical Investigation*, *52*(2), 441–453. https://doi.org/10.1172/JCI107201

Giraud, E., Cloeckaert, A., Kerboeuf, D. and Chaslus-Dancla, E. (2000). Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in Salmonella enterica serovar typhimurium. *Antimicrobial Agents and Chemotherapy*, *44*(5), 1223–1228. https://doi.org/10.1128/AAC.44.5.1223-1228.2000

Glasel, J. A. (1995). Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *BioTechniques*, *18*(1), 62–63.

Goetz, D. H., Holmes, M. A., Borregaard, N., Bluhm, M. E., Raymond, K. N. and Strong, R. K. (2002). The Neutrophil Lipocalin NGAL Is a Bacteriostatic Agent that Interferes with Siderophore-Mediated Iron Acquisition. *Molecular Cell*, *10*(5), 1033–1043. https://doi.org/10.1016/S1097-2765(02)00708-6

Gohin, I., Olivier, M., Lantier, I., Pépin, M. and Lantier, F. (1997). Analysis of the immune response in sheep efferent lymph during Salmonella abortusovis infection. *Veterinary Immunology and Immunopathology*, *60*(1–2), 111–130. https://doi.org/10.1016/S0165-2427(97)00090-1

Gomes, C., Ruiz-Roldán, L., Mateu, J., Ochoa, T. J. and Ruiz, J. (2019). Azithromycin resistance levels and mechanisms in Escherichia coli. *Scientific Reports*, *9*(1), 6089. https://doi.org/10.1038/S41598-019-42423-3

González-Torres, B., González-Gómez, J. P., Ramírez, K., Castro-del Campo, N., González-López, I., Garrido-Palazuelos, L. I., Chaidez, C. and Medrano-Félix, J. A. (2023). Population structure of the Salmonella enterica serotype Oranienburg reveals similar virulence, regardless of isolation years and sources. *Gene*, *851*, 146966. https://doi.org/10.1016/J.GENE.2022.146966

Gorivodsky, M., Zemlyak, I., Orenstein, H., Savion, S., Fein, A., Torchinsky, A. and Toder, V. (1998). TNF-α Messenger RNA and Protein Expression in the Uteroplacental Unit of Mice with Pregnancy Loss. *The Journal of Immunology*, *160*(9).

Graham, M. R., Virtaneva, K., Porcella, S. F., Barry, W. T., Gowen, B. B., Johnson, C. R., Wright, F. A. and Musser, J. M. (2005). Group A Streptococcus transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *The American Journal of Pathology*, *166*(2), 455–465. https://doi.org/10.1016/S0002-9440(10)62268-7

Grant, A. J., Foster, G. L., McKinley, T. J., Brown, S. P., Clare, S., Maskell, D. J. and Mastroeni, P. (2009). Bacterial growth rate and host factors as determinants of intracellular bacterial distributions in systemic Salmonella enterica infections. *Infection and Immunity*, *77*(12), 5608–5611. https://doi.org/10.1128/IAI.00827-09

Grieneisen, L., Dasari, M., Gould, T. J., … Blekhman, R. (2021). Gut microbiome heritability is near-universal but environmentally contingent. *Science*, *373*(6551), 181. https://doi.org/10.1126/SCIENCE.ABA5483

Grimont, P. A. D. and Weill, F.-X. (2006). WHO Collaborating Centre for Reference and Research on Salmonella ANTIGENIC FORMULAE OF THE SALMONELLA SEROVARS 2007 9th edition.

Guiney, D. G. and Fierer, J. (2011). The Role of the spv Genes in Salmonella Pathogenesis. *Frontiers in Microbiology*, *2*, 129. https://doi.org/10.3389/FMICB.2011.00129

Gulig, P. A. and Doyle, T. J. (1993). The Salmonella typhimurium Virulence Plasmid Increases the Growth Rate of Salmonellae in Mice. *Infection and Immunity*, *61*(2), 504–511.

Haddad, J. P. A., Dohoo, I. R. and VanLeewen, J. A. (2005). A review of Neospora caninum in dairy and beef cattle - a Canadian perspective. *The Canadian Veterinary Journal*, *46*(3), 230–243.

Haeger, J.-D., Hambruch, N., Dantzer, V., Hoelker, M., Schellander, K., Klisch, K. and Pfarrer, C. (2015). Changes in endometrial ezrin and cytokeratin 18 expression during bovine implantation and in caruncular endometrial spheroids in vitro. *Placenta*, *36*(8), 821–831. https://doi.org/10.1016/J.PLACENTA.2015.06.001

Haeger, J.-D., Hambruch, N. and Pfarrer, C. (2016). The bovine placenta in vivo and in vitro. *Theriogenology*, *86*(1), 306–312. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.043

Hall, G. A. and Jones, P. W. (1976). An Experimental Study of Salmonella Dublin Abortion in Cattle. *British Veterinary Journal*, *132*(1), 60–65. https://doi.org/10.1016/S0007-1935(17)34788-7

Hall, G. A. and Jones, P. W. (1977). A Study of the Pathogenesis of Experimental Salmonella Dublin abortion in cattle. *Journal of Comparative Pathology*, *87*, 53–65. https://doi.org/10.1016/0021-9975(77)90079-2

Hall, G. A. and Jones, P. W. (1979). Experimental oral infections of pregnant heifers with Salmonella dublin. *The British Veterinary Journal*, *135*(1), 75–82. https://doi.org/10.1016/S0007-1935(17)32991-3

Hall, G. A., Jones, P. W., Parsons, K. R., Chanter, N. and Aitken, M. M. (1979). Studies of The Virulence of Salmonella Dublin In Experimental Infections of Cattle and Rats. *British Veterinary Journal*, *135*(3), 243–248. https://doi.org/10.1016/S0007-1935(17)32883-X

Hall, G. A., Jones, P. W., Parsons, K. R., Young, E. R. and Aitken, M. M. (1980). The Haematology of Experimental Salmonella Dublin Infections of Pregnant Heifers. *British Veterinary Journal*, *136*(2), 182–189. https://doi.org/10.1016/S0007-1935(17)32342-4

Hambruch, N., Kumstel, S., Haeger, J.-D. and Pfarrer, C. (2017). Bovine placentomal heparanase and syndecan expression is related to placental maturation. *Placenta*, *57*, 42–51. https://doi.org/10.1016/J.PLACENTA.2017.06.006

Han, J., Lynne, A. M., David, D. E., Tang, H., Xu, J., Nayak, R., Kaldhone, P., Logue, C. M. and Foley, S. L. (2012). DNA Sequence Analysis of Plasmids from Multidrug Resistant Salmonella enterica Serotype Heidelberg Isolates. *PLoS ONE*, *7*(12), 51160. https://doi.org/10.1371/JOURNAL.PONE.0051160

Hantke, K., Nicholson, G., Rabsch, W. and Winkelmann, G. (2003). Salmochelins, siderophores of Salmonella enterica and uropathogenic Escherichia coli strains, are recognized by the outer membrane receptor IroN. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 3677–3682. https://doi.org/10.1073/PNAS.0737682100

Hashimoto, Y., Li, N., Yokoyama, H. and Ezaki, T. (1993). Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in Salmonella typhi. *Journal of Bacteriology*, *175*(14), 4456–4465. https://doi.org/10.1128/JB.175.14.4456-4465.1993

Haw, N. (1977). Salmonella Infections in Badgers. *British Veterinary Journal*, *133*, 526–529. https://doi.org/10.1016/S0007-1935(17)33996-9

Heithoff, D. M., Shimp, W. R., Lau, P. W., Badie, G., Enioutina, E. Y., Daynes, R. A., Byrne, B. A., House, J. K. and Mahan, M. J. (2008). Human Salmonella Clinical Isolates Distinct from Those of Animal Origin. *Applied and Environmental Microbiology*, *74*(6), 1757–1766. https://doi.org/10.1128/AEM.02740-07

Helms, M., Vastrup, P., Gerner-smidt, P. and Mølbak, K. (2003). Short and long term mortality associated with foodborne bacterial gastrointestinal infections: registry based study. *British Medical Journal*, *326*(7385), 357.

Henderson, K. and Mason, C. (2017). Diagnosis and control of Salmonella Dublin in dairy Herds. *In Practice*, *39*, 158–168. https://doi.org/10.1136/inp.j1160

Hilkens, C. M. U., Vermeulen, H., van Neerven, R. J. J., Snijdewint, F. G. M., Wierenga, E. A. and Kapsenber, M. L. (1995). Differential modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. *European Journal of Immunology*, *25*(1), 59–63. https://doi.org/10.1002/EJI.1830250112

Hinton, M. (1977). The diagnosis of salmonella abortion in cattle with particular reference to Salmonella dublin. A review. *Journal of Hygiene*, *79*(1), 25–38. https://doi.org/10.1017/S0022172400052815

Hirakawa, H., Nishino, K., Hirata, T. and Yamaguchi, A. (2003). Comprehensive Studies of Drug Resistance Mediated by Overexpression of Response Regulators of Two-Component Signal Transduction Systems in Escherichia coli. *Journal of Bacteriology*, *185*(6), 1851–1856. https://doi.org/10.1128/JB.185.6.1851-1856.2003

Hoffmann, B. and Schuler, G. (2002). The bovine placenta; a source and target of steroid hormones: observations during the second half of gestation. *Domestic Animal Endocrinology*, *23*(1–2), 309–320. https://doi.org/10.1016/S0739-7240(02)00166-2

Hoffmann, M., Pettengill, J. B., Gonzalez-Escalona, N., Miller, J., Ayers, S. L., Zhao, S., Allard, M. W., McDermott, P. F., Brown, E. W. and Monday, S. R. (2017). Comparative sequence analysis of multidrug-resistant incA/C plasmids from salmonella enterica. *Frontiers in Microbiology*, *8*, 1459. https://doi.org/10.3389/FMICB.2017.01459

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S. and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, *35*(4), 322–332. https://doi.org/10.1016/J.IJANTIMICAG.2009.12.011

Holschbach, C. L. and Peek, S. F. (2018). Salmonella in Dairy Cattle. *Veterinary Clinics of North America: Food Animal Practice*, *34*, 133–154. https://doi.org/10.1016/j.cvfa.2017.10.005

Hotson, A. G. and Schneider, D. S. (2015). Drosophila melanogaster natural variation affects growth dynamics of infecting Listeria monocytogenes. *G3: Genes, Genomes, Genetics*, *5*(12), 2593–2600. https://doi.org/10.1534/G3.115.022558

Hovingh, E. (2002). *Abortions in dairy cattle II: Diagnosing Abortion Problems*. *Virginia Cooperative Extension*.

Hovingh, E. (2009). *Abortion in dairy cattle I: Common causes of abortion*. *Virginia Cooperative Extension*.

Hu, B., Lara-Tejero, M., Kong, Q., Galá, J. E. and Liu, J. (2017). In Situ Molecular Architecture of the Salmonella Type III Secretion Machine. *Cell*, *168*, 1065–1074. https://doi.org/10.1016/j.cell.2017.02.022

Hughes, S., Poh, T. Y., Bumstead, N. and Kaiser, P. (2007). Re-evaluation of the chicken MIP family of chemokines and their receptors suggests that CCL5 is the prototypic MIP family chemokine, and that different species have developed different repertoires of both the CC chemokines and their receptors. *Developmental & Comparative Immunology*, *31*(1), 72–86. https://doi.org/10.1016/J.DCI.2006.04.003

Humphries, A. D., Townsend, S. M., Kingsley, R. A., Nicholson, T. L., Tsolis, R. M. and Bäumler, A. J. (2001). Role of fimbriae as antigens and intestinal colonization factors of Salmonella serovars. *FEMS Microbiology Letters*, *201*(2), 121–125. https://doi.org/10.1111/J.1574-6968.2001.TB10744.X

Iba, T., Hashiguchi, N., Nagaoka, I., Tabe, Y. and Murai, M. (2013). Neutrophil cell death in response to infection and its relation to coagulation. *Journal of Intensive Care*, *1*(1), 13. https://doi.org/10.1186/2052-0492-1-13

Jacobsen, A., Hendriksen, R. S., Aaresturp, F. M., Ussery, D. W. and Friis, C. (2011). The Salmonella enterica pan-genome. *Microbial Ecology*, *62*(3), 487–504. https://doi.org/10.1007/s00248-011-9880-1

Jiang, X., Rossanese, O. W., Brown, N. F., Kujat-choy, S., Galán, J. E., Finlay, B. B. and Brumell, J. H. (2004). The related effector proteins SopD and SopD2 from Salmonella enterica serovar Typhimurium contribute to virulence during systemic infection of mice. *Molecular Microbiology*, *54*(5), 1186–1198. https://doi.org/10.1111/j.1365-2958.2004.04344.x

Jiménez-Pelayo, L., García-Sánchez, M., Regidor-Cerrillo, J., Horcajo, P., Collantes-Fernández, E., Gómez-Bautista, M., Hambruch, N., Pfarrer, C. and Ortega-Mora, L. M. (2019a). Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with high and low virulence isolates of Neospora

caninum. *Parasites & Vectors*, *10*(1), 463. https://doi.org/10.1186/s13071-017-2409-9

Jiménez-Pelayo, L., García-Sánchez, M., Regidor-Cerrillo, J., Horcajo, P., Collantes-Fernández, E., Gómez-Bautista, M., Hambruch, N., Pfarrer, C. and Ortega-Mora, L. M. (2019b). Immune response profile of caruncular and trophoblast cell lines infected by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of Neospora caninum. *Parasites & Vectors*, *12*(1), 218. https://doi.org/10.1186/s13071-019-3466-z

Jones, B. D., Ghori, N. and Falkow, S. (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *The Journal of Experimental Medicine*, *180*(1), 15. https://doi.org/10.1084/JEM.180.1.15

Jones, M. A., Wigley, P., Page, K. L., Hulme, S. D. and Barrow, P. A. (2001). Salmonella enterica serovar Gallinarum requires the Salmonella pathogenicity island 2 type III secretion system but not the Salmonella pathogenicity island 1 type III secretion system for virulence in chickens. *Infection and Immunity*, *69*(9), 5471–5476. https://doi.org/10.1128/IAI.69.9.5471-5476.2001

Jones, T F, Ingram, L. A., Cieslak, P. R., Vugia, D. J., Tobin-D'Angelo, M., Hurd, S., Medus, C., Cronquist, A. and Angulo, F. J. (2008). Salmonellosis Outcomes Differ Substantially by Serotype. *The Journal of Infectious Diseases*, *198*(1), 109–114. https://doi.org/10.1086/588823

Jungi, T. W., Thöny, M., Brcic, M., Adler, B., Pauli, U. and Peterhans, E. (1996). Induction of Nitric Oxide Synthase in Bovine Mononuclear Phagocytes is Differentiation Stage-Dependent. *Immunobiology*, *195*(3), 385–400. https://doi.org/10.1016/S0171-2985(96)80054-4

Kalinski, P. (2012). Regulation of Immune Responses by Prostaglandin E2. *Journal of Immunology*, *188*(1), 21–28. https://doi.org/10.4049/JIMMUNOL.1101029

Kaliński, P., Hilkens, C., Snijders, A., Snijdewint, F. and Kapsenberg, M. (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *Journal of Immunology*, *1*(159), 28–35.

Kämmer, P., McNamara, S., Wolf, T., Conrad, T., Allert, S., Gerwien, F., Hünniger, K., Kurzai, O., Guthke, R., Hube, B., Linde, J. and Brunke, S. (2020). Survival Strategies of Pathogenic Candida Species in Human Blood Show Independent and Specific Adaptations. *MBio*, *11*(5), 1–21. https://doi.org/10.1128/MBIO.02435-20

Kendrick, J. W. (1976). Bovine viral diarrhea virus-induced abortion. *Theriogenology*, *5*(3), 91–93. https://doi.org/10.1016/0093-691X(76)90028-5

Kent, E., Okafor, C., Caldwell, M., Walker, T., Whitlock, B. and Lear, A. (2021). Control of Salmonella Dublin in a bovine dairy herd. *Journal of Veterinary Internal Medicine*, *35*(4), 2075. https://doi.org/10.1111/JVIM.16191

Kerr, K., Entrican, G., McKeever, D. and Longbottom, D. (2005). Immunopathology of Chlamydophila abortus infection in sheep and mice. *Research in Veterinary Science*, *78*(1), 1–7. https://doi.org/10.1016/J.RVSC.2004.08.004

King, G. J., Atkinson, B. A. and Robertson, H. A. (1980). Development of the bovine placentome from days 20 to 29 of gestation. *Journal of Reproduction and Fertility*, (59), 95–100. https://doi.org/10.1530/jrf.0.0590095

Kirchner, M. J., Liebana, E., McLaren, I., Clifton-Hadley, F. A., Wales, A. D. and Davies, R. H. (2012). Comparison of the environmental survival characteristics of Salmonella Dublin and Salmonella Typhimurium. *Veterinary Microbiology*, *159*(3–4), 509–514. https://doi.org/10.1016/j.vetmic.2012.04.009

Kirkwood, M., Vohra, P., Bawn, M., Thilliez, G., Pye, H., Tanner, J., Chintoan-Uta, C., Branchu, P., Petrovska, L., Dallman, T., Hall, N., Stevens, M. P. and Kingsley, R. A. (2021). Ecological niche adaptation of Salmonella Typhimurium U288 is associated with altered pathogenicity and reduced zoonotic potential. *Communications Biology 2021 4:1*, *4*(1), 1–15. https://doi.org/10.1038/s42003-021-02013-4

Klose, C., Scuda, N., Ziegler, T., Eisenberger, D., Hanczaruk, M. and Riehm, J. M. (2022). Whole-genome investigation of Salmonella Dublin considering mountain pastures as reservoirs in Southern Bavaria, Germany. *Microorganisms*, *10*(5), 885. https://doi.org/10.3390/MICROORGANISMS10050885

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M. and Steele-Mortimer, O. (2010). Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(41), 17733–17738. https://doi.org/10.1073/PNAS.1006098107

Knuff, K. and Finlay, B. B. (2017). What the SIF Is Happening—The Role of Intracellular Salmonella-Induced Filaments. *Frontiers in Cellular and Infection Microbiology*, *25*(7), 335. https://doi.org/10.3389/FCIMB.2017.00335

Koczerka, M., Douarre, P.-E., Kempf, F., Holbert, S., Mistou, M.-Y., Grépinet, O. and Virlogeux-Payant, I. (2021). The Invasin and Complement-Resistance Protein Rck of Salmonella is More Widely Distributed than Previously Expected. *Microbiology Spectrum*, *9*(2), e0145721. https://doi.org/10.1128/SPECTRUM.01457-21

Krishnan, L., Cuilbert, L. J., Wegmann, T. G., Belosevic, M. and Mosmann3, T. R. (1996). T Helper 1 Response Against Leishmania major in Pregnant C57BL/6 Mice Increases Implantation Failure and Fetal Resorptions. Correlation with Increased IFN-y and TNF and Reduced 11-10 Production by Placental Cells. *Journal of Immunology*, *156*(2), 653–662. Retrieved from http://www.jimmunol.org/

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J. E. and Aizawa, S. I. (1998). Supramolecular Structure of the Salmonella typhimurium Type III Protein Secretion System. *Science*, *280*(5363), 602–605. https://doi.org/10.1126/SCIENCE.280.5363.602

Kumar, S. and Varela, M. F. (2012). Biochemistry of Bacterial Multidrug Efflux Pumps. *International Journal of Molecular Sciences*, *13*(4), 4484. https://doi.org/10.3390/IJMS13044484

Kuzminska-Bajor, M., Grzymajlo, K. and Ugorski, M. (2015). Type 1 fimbriae are important factors limiting the dissemination and colonization of mice by Salmonella Enteritidis and contribute to the induction of intestinal inflammation during Salmonella invasion. *Frontiers in Microbiology*, *6*, 276. https://doi.org/10.3389/FMICB.2015.00276

Landsverk, T., Lium, B., Matovelo, J. A., Liven, E. and Nordstoga, K. (1990). Peyer's patches in experimental Salmonella dublin infection in calves. Microvascular and epithelial changes contributing to atrophy of lymphoid follicles. *APMIS*, *98*(3), 255–268. https://doi.org/10.1111/J.1699-0463.1990.TB01030.X

Langridge, G. C., Fookes, M., Connor, T. R., … Thomson, N. R. (2015). Patterns of genome evolution that have accompanied host adaptation in Salmonella. *PNAS*, *112*(3), 863–868. https://doi.org/10.1073/pnas.1416707112

Langridge, G. C., Nair, S. and Wain, J. (2009). Nontyphoidal Salmonella Serovars Cause Different Degrees of Invasive Disease Globally. *The Journal of Infectious Diseases*, *199*(4), 602–603. https://doi.org/10.1086/596208

Lara-Tejero, M., Kato, J., Wagner, S., Liu, X. and Galán, J. E. (2011). A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems. *Source: Science, New Series*, *331*(6021), 1188–1191. https://doi.org/10.1126/science.ll63732

LaRock, D. L., Chaudhary, A. and Miller, S. I. (2015). Salmonellae interactions with host processes. *Nature Reviews Microbiology*, *13*, 191–205. https://doi.org/10.1038/nrmicro3420

Laster, S. M., Wood, J. G. and Gooding, L. R. (1988). Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *The Journal of Immunology*, *141*(8), 2629–2634.

Laupland, K. B., Schønheyder, H. C., Kennedy, K. J., Lyytikäinen, O., Valiquette, L., Galbraith, J. and Collignon, P. (2010). Salmonella enterica bacteraemia: A multi-national population-based cohort study. *BMC Infectious Diseases*, *10*, 95. https://doi.org/10.1186/1471-2334-10-95

Leaver, H. A., Howie, A., Aitken, I. D., Appleyard, B. W., Anderson, I. E., Jones, G., Hay, L. A., Williams, G. E. and Buxton, D. (1989). Changes in Progesterone, Oestradiol 17β, and Intrauterine Prostaglandin E2 during Late Gestation in Sheep Experimentally Infected with an Ovine Abortion Strain of Chlamydia psittaci. *Microbiology*, *135*(3), 565–573. https://doi.org/10.1099/00221287-135-3-565

Leon-Sicairos, N., Reyes-Cortes, R., Guadrón-Llanos, A. M., Madueña-Molina, J., Leon-Sicairos, C. and Canizalez-Román, A. (2015). Strategies of Intracellular Pathogens for Obtaining Iron

from the Environment. *BioMed Research International*, *2015*, 476354. https://doi.org/10.1155/2015/476534

Lesnick, M. L., Reiner, N. E., Fierer, J. and Guiney, D. G. (2001). The Salmonella spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Molecular Microbiology*, *39*(6), 1464–1470. https://doi.org/10.1046/J.1365-2958.2001.02360.X

Letunic, I. and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, *23*(1), 127–128. https://doi.org/10.1093/BIOINFORMATICS/BTL529

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G. and Guan, L. L. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome*, *7*(1), 1–17. https://doi.org/10.1186/S40168-019-0699-1

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., Chen, S., Zhou, J. M. and Shao, F. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. *Science*, *315*(5814), 1000–1003. https://doi.org/10.1126/SCIENCE.1138960

Li, J., Smith, N. H., Nelson, K., Crichton, P. B., Old, D. C., Whittam, T. S. and Selander, R. K. (1993). Evolutionary origin and radiation of the avian-adapted non-motile salmonellae. *Journal of Medical Microbiology*, *38*(2), 129–139. https://doi.org/10.1099/00222615-38-2-129

Liaquat, S., Sarwar, Y., Ali, A. and Haque, A. (2015). Comparative growth analysis of capsulated (Vi+) and acapsulated (Vi-) Salmonella typhi isolates in human blood. *EXCLI Journal*, *14*, 213. https://doi.org/10.17179/EXCLI2014-674

Libby, S. J., Adams, L. G., Ficht, T. A., Allen, C., Whitford, H. A., Buchmeier, N. A., Bossie, S. and Guiney, D. G. (1997). The spy genes on the Salmonella dublin virulence plasmid are required for severe enteritis and systemic infection in the natural host. *Infection and Immunity*, *65*(5), 1786–1792.

Lilleengen, K. (1948). Typing Salmonella by means of bacteriophage. *Acta Pathologica et Microbiologica Scandinavica*, *77*, 11–125.

Liss, V., Swart, A. L., Kehl, A., Hermanns, N., Zhang, Y., Chikkaballi, D., Böhles, N., Deiwick, J. and Hensel, M. (2017). Salmonella enterica Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition. *Cell Host & Microbe*, *21*(3), 390–402. https://doi.org/10.1016/J.CHOM.2017.02.005

Liu, B., Zheng, D., Jin, Q., Chen, L. and Yang, J. (2019). VFDB 2019: A comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*, *47*(D1), D687–D692. https://doi.org/10.1093/nar/gky1080

Liu, B., Zheng, D., Zhou, S., Chen, L. and Yang, J. (2022). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucleic Acids Research*, *50*(D1), D912–D917. https://doi.org/10.1093/NAR/GKAB1107

Liu, G. R., Edwards, K., Eisenstark, A., Fu, Y. M., Liu, W. Q., Sanderson, K. E., Johnston, R. N. and Liu, S. L. (2003). Genomic Diversification among Archival Strains of Salmonella enterica Serovar Typhimurium LT7. *Journal of Bacteriology*, *185*(7), 2131. https://doi.org/10.1128/JB.185.7.2131-2142.2003

Liu, J., Keelan, P., Bennett, P. M. and Enne, V. I. (2009). Characterization of a novel macrolide efflux gene, mef(B), found linked to sul3 in porcine Escherichia coli. *The Journal of Antimicrobial Chemotherapy*, *63*(3), 423–426. https://doi.org/10.1093/JAC/DKN523

Liu, L., Ge, D., Ma, L., Mei, J., Liu, S., Zhang, Q., Ren, F., Liao, H., Pu, Q., Wang, T. and You, Z. (2012). Interleukin-17 and Prostaglandin E2 Are Involved in Formation of an M2 Macrophage-Dominant Microenvironment in Lung Cancer. *Journal of Thoracic Oncology*, *7*(7), 1091–1100. https://doi.org/10.1097/JTO.0B013E3182542752

Liu, T., Zhang, L., Joo, D. and Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*, 17023. https://doi.org/10.1038/sigtrans.2017.23

Liu, Y., Zhang, D. F., Zhou, X., Xu, L., Zhang, L. and Shi, X. (2017). Comprehensive analysis reveals two distinct evolution patterns of Salmonella flagellin gene clusters. *Frontiers in Microbiology*, *8*, 2604. https://doi.org/10.3389/FMICB.2017.02604

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/METH.2001.1262

Livermore, D. M. (1995). beta-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*, *8*(4), 557–584. https://doi.org/10.1128/CMR.8.4.557

Llana, M. N., Sarnacki, S. H., Del Rosario Aya Castañeda, M., Pustovrh, M. C., Gartner, A. S., Buzzola, F. R., Cerquetti, M. C. and Giacomodonato, M. N. (2014). Salmonella enterica serovar Enteritidis enterocolitis during late stages of gestation induces an adverse pregnancy outcome in the murine model. *PloS One*, *9*(11), e111282. https://doi.org/10.1371/JOURNAL.PONE.0111282

Longbottom, D. and Coulter, L. J. (2003). Animal Chlamydioses and Zoonotic Implications. *Journal of Comparative Pathology*, *128*(4), 217–244. https://doi.org/10.1053/JCPA.2002.0629

López-Garrido, J. and Casadesús, J. (2012). Crosstalk between Virulence Loci: Regulation of Salmonella enterica Pathogenicity Island 1 (SPI-1) by Products of the std Fimbrial Operon. *PLOS ONE*, *7*(1), e30499. https://doi.org/10.1371/JOURNAL.PONE.0030499

Lostroh, C. P. and Lee, C. A. (2001). The HilA Box and Sequences outside It Determine the Magnitude of HilA-Dependent Activation of PprgH from Salmonella Pathogenicity Island 1. *Journal of Bacteriology*, *183*(16), 4876. https://doi.org/10.1128/JB.183.16.4876-4885.2001

Lupolova, N., Dallman, T. J., Holden, N. J. and Gally, D. L. (2017). Patchy promiscuity: machine learning applied to predict the host specificity of Salmonella enterica and Escherichia coli. *Microbial Genomics*, *3*(10), e000135. https://doi.org/10.1099/MGEN.0.000135

Mackenzie, K. D., Wang, Y., Musicha, P., Hansen, E. G., Palmer, M. B., Herman, D. J., Feasey, N. A. and White, A. P. (2019). Parallel evolution leading to impaired biofilm formation in invasive Salmonella strains. *PLoS Genetics*, *15*(6), e1008233. https://doi.org/10.1371/JOURNAL.PGEN.1008233

Mailliard, R. B., Alber, S. M., Shen, H., Watkins, S. C., Kirkwood, J. M., Herberman, R. B. and Kalinski, P. (2005). IL-18–induced CD83+CCR7+ NK helper cells. *The Journal of Experimental Medicine*, *202*(7), 941. https://doi.org/10.1084/JEM.20050128

Makino, F., Shen, D., Kajimura, N., Kawamoto, A., Pissaridou, P., Oswin, H., Pain, M., Murillo, I., Namba, K. and Blocker, A. J. (2016). The Architecture of the Cytoplasmic Region of Type III Secretion Systems. *Nature Scientific Reports*, *6*, 33341. https://doi.org/10.1038/srep33341

Mangat, C. S., Bekal, S., Irwin, R. J. and Mulvey, M. R. (2017). A Novel Hybrid Plasmid Carrying Multiple Antimicrobial Resistance

and Virulence Genes in Salmonella enterica Serovar Dublin. *Antimicrobial Agents and Chemotherapy*, *61*(6), e02601–e02616. https://doi.org/10.1128/AAC.02601-16

Martínez-Colón, G. J. and Moore, B. B. (2018). Prostaglandin E2 as a Regulator of Immunity to Pathogens. *Pharmacology & Therapeutics*, *185*, 135–146. https://doi.org/10.1016/J.PHARMTHERA.2017.12.008

Mastroeni, P., Skepper, J. N. and Hormaeche, C. E. (1995). Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary Salmonella infections. *Infection and Immunity*, *63*(9), 3674–3682. https://doi.org/10.1128/iai.63.9.3674-3682.1995

McArthur, A. G., Waglechner, N., Nizam, F., … Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3348–3357. https://doi.org/10.1128/AAC.00419-13

McCormick, B. A., Miller, S. I., Carnes, D. and Madara, J. L. (1995). Transepithelial signaling to neutrophils by salmonellae: a novel virulence mechanism for gastroenteritis. *Infection and Immunity*, *63*(6), 2302. https://doi.org/10.1128/IAI.63.6.2302-2309.1995

McCormick, Beth A., Hofman, P. M., Kim, J., Carnes, D. K., Miller, S. I. and Madara, J. L. (1995). Surface attachment of Salmonella typhimurium to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. *The Journal of Cell Biology*, *131*(6), 1599. https://doi.org/10.1083/JCB.131.6.1599

McCuddin, Z. P., Carlson, S. A., Rasmussen, M. A. and Franklin, S. K. (2006). Klebsiella to Salmonella gene transfer within rumen protozoa: Implications for antibiotic resistance and rumen defaunation. *Veterinary Microbiology*, *114*(3–4), 275–284. https://doi.org/10.1016/J.VETMIC.2005.12.004

McGhie, E. J., Hayward, R. D. and Koronakis, V. (2004). Control of Actin Turnover by a Salmonella Invasion Protein. *Molecular Cell*, *13*(4), 497–510. https://doi.org/10.1016/S1097-2765(04)00053-X

McGourty, K., Thurston, T. L., Matthews, S. A., Pinaud, L., Mota, L. J. and Holden, D. W. (2012). Salmonella inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function. *Science*, *338*(6109), 963–967. https://doi.org/10.1126/SCIENCE.1227037

McMillan, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., Barrett, J. B., Jackson, C. R., Wasilenko, J. L., Simmons, M., Tillman, G. E., McClelland, M. and Frye, J. G. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in salmonella enterica associated with United States food animals. *Frontiers in Microbiology*, *10*, 832. https://doi.org/10.3389/FMICB.2019.00832

Mereghetti, L., Sitkiewicz, I., Green, N. M. and Musser, J. M. (2008). Extensive adaptive changes occur in the transcriptome of Streptococcus agalactiae (group B streptococcus) in response to incubation with human blood. *PloS One*, *3*(9), e3143. https://doi.org/10.1371/JOURNAL.PONE.0003143

Miles, A. A., Misra, S. S. and Irwin, J. 0. (1938). The Estimation of the Bactericidal Power of the Blood. *Journal of Hygeine*, *38*(6), 732–749. https://doi.org/10.1017/s002217240001158x

Mirold, S., Ehrbar, K., Weissmüller, A., Tschäpe, H., Rüssmann, H. and Hardt, W. (2001). Salmonella host cell invasion emerged by acquisition of a mosaic of separate genetic elements, including Salmonella pathogenicity island 1 (SPI1), SPI5, and sopE2. *Journal of Bacteriology*, *183*(7), 2348–2358. https://doi.org/10.1128/JB.183.7.2348-2358.2001

Miyoshi, M. and Sawamukai, Y. (2004). Specific Localization of Macrophages in Pregnant Bovine Caruncles. *Reproduction in Domestic Animals*, *39*(3), 125–128. https://doi.org/10.1111/J.1439-0531.2004.00484.X

Mohammed, M. and Cormican, M. (2016). Whole genome sequencing provides insights into the genetic determinants of invasiveness in Salmonella Dublin. *Epidemiology & Infection*, *144*(11), 2430–2439. https://doi.org/10.1017/S0950268816000492

Mohammed, M., Le Hello, S., Leekitcharoenphon, P. and Hendriksen, R. (2017). The invasome of Salmonella Dublin as revealed by whole genome sequencing. *BMC Infectious Diseases*, *17*(1), 544. https://doi.org/10.1186/s12879-017-2628-x

Mohammed, Manal and Thapa, S. (2020). Evaluation of WGS-subtyping methods for epidemiological surveillance of foodborne salmonellosis. *One Health Outlook*, *2*(1), 1–15. https://doi.org/10.1186/S42522-020-00016-5

Mohammed, Manal, Vignaud, M.-L. and Cadel-Six, S. (2019). Whole-Genome Sequences of Two Salmonella enterica Serovar Dublin Strains That Harbor the viaA, viaB, and ompB Loci of the Vi Antigen. *Microbiology Resource Announcements*, *8*(14), e00028. https://doi.org/10.1128/MRA.00028-19

Monack, D. M., Hersh, D., Ghori, N., Bouley, D., Zychlinsky, A. and Falkow, S. (2000). Salmonella Exploits Caspase-1 to Colonize Peyer's Patches in a Murine Typhoid Model. *The Journal of Experimental Medicine*, *192*(2), 249. https://doi.org/10.1084/JEM.192.2.249

Montagne, A., Menanteau, P., Boivin, R., Bernard, S., Lantier, F. and Lalmanach, A. C. (2001). Cytokine gene expression in lymph node and spleen of sheep in response to Salmonella infection by two serotypes displaying different host specificity. *Veterinary*

Immunology and Immunopathology, *82*(3–4), 257–272. https://doi.org/10.1016/S0165-2427(01)00366-X

Moreno Switt, A. I., den Bakker, H. C., Cummings, C. A., Rodriguez-Rivera, L. D., Govoni, G., Raneiri, M. L., Degoricija, L., Brown, S., Hoelzer, K., Peters, J. E., Bolchacova, E., Furtado, M. R. and Wiedmann, M. (2012). Identification and characterization of novel Salmonella mobile elements involved in the dissemination of genes linked to virulence and transmission. *PLoS ONE*, *7*(7), e41247. https://doi.org/10.1371/journal.pone.0041247

Morimoto, Y. V. and Minamino, T. (2014). Structure and Function of the Bi-Directional Bacterial Flagellar Motor. *Biomolecules*, *4*(1), 217. https://doi.org/10.3390/BIOM4010217

Mu, Y., Li, R., Du, P., Zhang, P., Li, Y., Cui, S., Fanning, S. and Bai, L. (2022). Genomic Epidemiology of ST34 Monophasic Salmonella enterica Serovar Typhimurium from Clinical Patients from 2008 to 2017 in Henan, China. *Engineering*, *15*, 34–44. https://doi.org/10.1016/J.ENG.2022.05.006

Mulder, D. T., Cooper, C. A. and Coombes, B. K. (2012). Type VI Secretion System-Associated Gene Clusters Contribute to Pathogenesis of Salmonella enterica Serovar Typhimurium. *Infection and Immunity*, *80*(6), 1996. https://doi.org/10.1128/IAI.06205-11

Nagy, T. A., Moreland, S. M., Andrews-Polymenis, H. and Detweiler, C. S. (2013). The Ferric Enterobactin Transporter Fep Is Required for Persistent Salmonella enterica Serovar Typhimurium Infection. *Infection and Immunity*, *81*(11), 4063. https://doi.org/10.1128/IAI.00412-13

National Institute for Health and Care Excellence. (2022). Gastro-intestinal system infections, antibacterial therapy - Treatment summary. Retrieved April 18, 2022, from

https://bnf.nice.org.uk/treatment-summary/gastro-intestinal-system-infections-antibacterial-therapy.html

National Library of Medicine and National Center for Biotechnology Information. (1988). Protein [Internet].

National Library of Medicine and National Center for Biotechnology Information. (2004). Gene [Internet]. Retrieved January 8, 2021, from https://www.ncbi.nlm.nih.gov/gene/

Navarro, J. A., García De La Fuente, J. N., Sánchez, J., Martínez, C. M., Buendía, A. J., Gutiérrez-Martín, C. B., Rodriguez-Ferri, E. F., Ortega, N. and Salinas, J. (2004). Kinetics of infection and effects on the placenta of Chlamydophila abortus in experimentally infected pregnant ewes. *Veterinary Pathology*, *41*(5), 498–505. https://doi.org/10.1354/VP.41-5-498

Neysens, P., Messens, W., Gevers, D., Swings, J. and De Vuyst, L. (2003). Biphasic kinetics of growth and bacteriocin production with Lactobacillus amylovorus DCE 471 occur under stress conditions. *Microbiology*, *149*(4), 1073–1082. https://doi.org/10.1099/mic.0.25880-0

Nielsen, L. R. (2013a). Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of Salmonella Dublin in cattle. *Veterinary Microbiology*, *162*(1), 1–9. https://doi.org/10.1016/j.vetmic.2012.08.003

Nielsen, L. R. (2013b). Salmonella Dublin faecal excretion probabilities in cattle with different temporal antibody profiles in 14 endemically infected dairy herds. *Epidemiology and Infection*, *141*(9), 1937–1944. https://doi.org/10.1017/S0950268812002579

Nielsen, L. R. and Dohoo, I. (2012). Survival analysis of factors affecting incidence risk of Salmonella Dublin in Danish dairy herds during a 7-year surveillance period. *Preventive Veterinary*

Medicine, *107*(3–4), 160–169. https://doi.org/10.1016/j.prevetmed.2012.06.002

Nielsen, L. R., Schukken, Y. H., Gröhn, Y. T. and Ersbøll, A. K. (2004). Salmonella Dublin infection in dairy cattle: Risk factors for becoming a carrier. *Preventive Veterinary Medicine*, *65*(1–2), 47–62. https://doi.org/10.1016/j.prevetmed.2004.06.010

Nielsen, T. D., Kudahl, A. B., Østergaard, S. and Nielsen, L. R. (2013). Gross margin losses due to Salmonella Dublin infection in Danish dairy cattle herds estimated by simulation modelling. *Preventive Veterinary Medicine*, *111*(1–2), 51–62. https://doi.org/10.1016/j.prevetmed.2013.03.011

Nikaido, H., Basina, M., Nguyen, V. and Rosenberg, E. Y. (1998). Multidrug Efflux Pump AcrAB of Salmonella typhimurium Excretes Only Those β-Lactam Antibiotics Containing Lipophilic Side Chains. *Journal of Bacteriology*, *180*(17), 4686. https://doi.org/10.1128/JB.180.17.4686-4692.1998

Nishino, K., Latifi, T. and Groisman, E. A. (2006). Virulence and drug resistance roles of multidrug efflux systems of Salmonella enterica serovar Typhimurium. *Molecular Microbiology*, *59*(1), 126–141. https://doi.org/10.1111/J.1365-2958.2005.04940.X

Nitta, A., Shirasuna, K., Haneda, S., Matsui, M., Shimizu, T., Matsuyama, S., Kimura, K., Bollwein, H. and Miyamoto, A. (2011). Possible involvement of IFNT in lymphangiogenesis in the corpus luteum during the maternal recognition period in the cow. *Reproduction*, *142*(6), 879–892. https://doi.org/10.1530/REP-11-0157

Njau, F., Wittkop, U., Rohde, M., Haller, H., Klos, A. and Wagner, A. D. (2009). In vitro neutralization of tumor necrosis factor-alpha during Chlamydia pneumoniae infection impairs dendritic cells maturation/function and increases chlamydial progeny. *FEMS*

Immunology and Medical Microbiology, *55*(2), 215–225. https://doi.org/10.1111/J.1574-695X.2008.00512.X

NOAH Compendium. (2017). NOAH Compendium | Bovivac S. Retrieved May 22, 2019, from http://www.noahcompendium.co.uk/?id=-454340

Noakes, D., Parkinson, T. and England, G. (2009). *Veterinary Reproduction and Obstetrics*.

Norris, M. H., Zincke, D., Leiser, O. P., Kreuzer, H., Hadfied, T. L. and Blackburn, J. K. (2020). Laboratory strains of Bacillus anthracis lose their ability to rapidly grow and sporulate compared to wildlife outbreak strains. *PLoS ONE*, *15*(1), e0228270. https://doi.org/10.1371/JOURNAL.PONE.0228270

Ochman, H., Soncinit, F. C., Solomont, F. and Groismantt, E. A. (1996). Identification of a pathogenicity island required for Salmonella survival in host cells. *Proceedings of the National Academy of Sciences of the United States of America.*, *93*, 7800–7804.

Off J Eur Union. EU Council. Council Regulation (EC) No 1/2005 of 22 December 2004 on the Protection of Animals during Transport and Related Operations and Amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97 (2005).

Ogushi, K. I., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., Takahashi, A., Asakura, H., Makino, S. I., Hojo, H., Nakahara, Y., Ohsaki, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Moss, J. and Hirayama, T. (2001). Salmonella enteritidis FliC (Flagella Filament Protein) Induces Human β-Defensin-2 mRNA Production by Caco-2 Cells. *Journal of Biological Chemistry*, *276*(32), 30521–30526. https://doi.org/10.1074/JBC.M011618200

Okusu, H., Ma, D. and Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia

coli multiple-antibiotic-resistance (Mar) mutants. *Journal of Bacteriology*, *178*(1), 306–308. https://doi.org/10.1128/JB.178.1.306-308.1996

Oliveira, L. J., Mansourri-Attia, N., Fahey, A. G., Browne, J., Forde, N., Roche, J. F., Lonergan, P. and Fair, T. (2013). Characterization of the Th Profile of the Bovine Endometrium during the Oestrous Cycle and Early Pregnancy. *PLoS ONE*, *8*(10), 75571. https://doi.org/10.1371/JOURNAL.PONE.0075571

Olsen, J. E., Hoegh-Andersen, K. H., Casadesús, J., Rosenkranzt, J., Chadfield, M. S. and Thomsen, L. E. (2013). The role of flagella and chemotaxis genes in host pathogen interaction of the host adapted Salmonella enterica serovar Dublin compared to the broad host range serovar S. Typhimurium. *BMC Microbiology*, *13*(1), 1–11. https://doi.org/10.1186/1471-2180-13-67

O'Neill, J. (2014). *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*.

O'Neill, J. (2016). *Tackling Drug-resistant infections globally: Final report and recommendations on antimicrobial resistance*.

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., Fookes, M., Falush, D., Keane, J. A. and Parkhill, J. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics*, *31*(22), 3691–3693. https://doi.org/10.1093/BIOINFORMATICS/BTV421

Parisi, A., Crump, J. A., Glass, K., Howden, B. P., Furuya-Kanamori, L., Vilkins, S., Gray, D. J. and Kirk, M. D. (2018). Health Outcomes from Multidrug-Resistant Salmonella Infections in High-Income Countries: A Systematic Review and Meta-Analysis. *Open Forum Infectious Diseases*, *15*(7), S286. https://doi.org/10.1089/FPD.2017.2403

Paudyal, N., Pan, H., Elbediwi, M., Zhou, X., Peng, X., Li, X., Fang, W. and Yue, M. (2019). Characterization of Salmonella Dublin isolated from bovine and human hosts. *BMC Microbiology*, *19*(1), 226. https://doi.org/10.1186/s12866-019-1598-0

Pecoraro, H. L., Thompson, B. and Duhamel, G. E. (2017). Histopathology case definition of naturally acquired *Salmonella enterica* serovar Dublin infection in young Holstein cattle in the northeastern United States. *Journal of Veterinary Diagnostic Investigation*, *29*(6), 860–864. https://doi.org/10.1177/1040638717712757

Penders, J., Stobberingh, E. E., Savelkoul, P. H. M. and Wolffs, P. F. G. (2013). The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in Microbiology*, *4*, 87. https://doi.org/10.3389/FMICB.2013.00087

Peter, A. T. (2013). Bovine placenta: A review on morphology, components, and defects from terminology and clinical perspectives. *Theriogenology*, *80*(7), 693–705. https://doi.org/10.1016/J.THERIOGENOLOGY.2013.06.004

Pickard, D., Wain, J., Baker, S., Line, A., Chohan, S., Fookes, M., Barron, A., Gaora, P. Ó., Chabalgoity, J. A., Thanky, N., Scholes, C., Thomson, N., Quail, M., Parkhill, J. and Dougan, G. (2003). Composition, Acquisition, and Distribution of the Vi Exopolysaccharide-Encoding Salmonella enterica Pathogenicity Island SPI-7. *Journal of Bacteriology*, *185*(17), 5055. https://doi.org/10.1128/JB.185.17.5055-5065.2003

Pitts, M. G. and D'Orazio, S. E. F. (2019). Prostaglandin E 2 Inhibits the Ability of Neutrophils to Kill Listeria monocytogenes. *The Journal of Immunology*, *202*(12), 3474–3482. https://doi.org/10.4049/JIMMUNOL.1900201

Ponnusamy, P., Ronald, B. S. M., Kumar, M. R., Anandachitra, M. and Manickam, R. (2017). International journal of science,

environment and technology. *International Journal of Science, Environment and Technology*, *6*(1), 284–287.

Public Health England. (2018). Salmonella data 2007 to 2016.

Pullinger, G. D., Dziva, F., Charleston, B., Wallis, T. S. and Stevens, M. P. (2008). Identification of Salmonella enterica serovar dublin-specific sequences by subtractive hybridization and analysis of their role in intestinal colonization and systemic translocation in cattle. *Infection and Immunity*, *76*(11), 5310–5321. https://doi.org/10.1128/IAI.00960-08

Pullinger, G. D., Paulin, S. M., Charleston, B., Watson, P. R., Bowen, A. J., Dziva, F., Morgan, E., Villarreal-Ramos, B., Wallis, T. S. and Stevens, M. P. (2007). Systemic translocation of Salmonella enterica serovar Dublin in cattle occurs predominantly via efferent lymphatics in a cell-free niche and requires type III secretion system 1 (T3SS-1) but not T3SS-2. *Infection and Immunity*, *75*(11), 5191–5199. https://doi.org/10.1128/IAI.00784-07

Quinn, H. E., Ellis, J. T. and Smith, N. C. (2002). Neospora caninum: A cause of immune-mediated failure of pregnancy? *Trends in Parasitology*, *18*(9), 391–394. https://doi.org/10.1016/S1471-4922(02)02324-3

Qureshi, T., Templeton, J. W. and Adams, L. G. (1996). Intracellular survival of Brucella abortus, Mycobacterium bovis BCG, Salmonella dublin, and Salmonella typhimurium in macrophages from cattle genetically resistant to Brucella abortus. *Veterinary Immunology and Immunopathology*, *50*(1–2), 55–65. https://doi.org/10.1016/0165-2427(95)05492-8

Raffatellu, M., George, M. D., Akiyama, Y., Hornsby, M. J., Nuccio, S. P., Paixao, T. A., Butler, B. P., Chu, H., Santos, R. L., Berger, T., Mak, T. W., Tsolis, R. M., Bevins, C. L., Solnick, J. V., Dandekar, S. and Bäumler, A. J. (2009). Lipocalin-2 Resistance Confers an

Advantage to Salmonella enterica Serotype Typhimurium for Growth and Survival in the Inflamed Intestine. *Cell Host and Microbe*, *5*(5), 476–486. https://doi.org/10.1016/J.CHOM.2009.03.011

Rakov, A. V, Mastriani, E., Liu, S.-L. and Schifferli, D. M. (2019). Association of Salmonella virulence factor alleles with intestinal and invasive serovars. *BMC Genomics*, *20*(1), 429. https://doi.org/10.1186/s12864-019-5809-8

Ramsden, A. E., Holden, D. W. and Mota, L. J. (2007). Membrane dynamics and spatial distribution of Salmonella-containing vacuoles. *Trends in Microbiology*, *15*(11), 516–524. https://doi.org/10.1016/j.tim.2007.10.002

Rappl, C., Deiwick, J. and Hensel, M. (2003). Acidic pH is required for the functional assembly of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *FEMS Microbiology Letters*, *226*(2), 363–372. https://doi.org/10.1016/S0378-1097(03)00638-4

Raymond, K. N., Dertz, E. A. and Kim, S. S. (2003). Enterobactin: An archetype for microbial iron transport. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 3584. https://doi.org/10.1073/PNAS.0630018100

Reen, F. J., Boyd, E. F., Porwollik, S., Murphy, B. P., Gilroy, D., Fanning, S. and McClelland, M. (2005). Genomic Comparisons of Salmonella enterica Serovar Dublin, Agona, and Typhimurium Strains Recently Isolated from Milk Filters and Bovine Samples from Ireland, Using a Salmonella Microarray. *Applied and Environmental Microbiology*, *71*(3), 1616. https://doi.org/10.1128/AEM.71.3.1616-1625.2005

Regidor-Cerrillo, J., Arranz-Solís, D., Benavides, J., Gómez-Bautista, M., Castro-Hermida, J. A., Mezo, M., Pérez, V., Ortega-Mora, L. M. and González-Warleta, M. (2014). Neospora caninum infection

during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. *Veterinary Research*, *45*(1), 10. https://doi.org/10.1186/1297-9716-45-10

Rehman, T., Yin, L., Latif, M. B., Chen, J., Wang, K., Geng, Y., Huang, X., Abaidullah, M., Guo, H. and Ouyang, P. (2019). Adhesive mechanism of different Salmonella fimbrial adhesins. *Microbial Pathogenesis*, *137*, 103748. https://doi.org/10.1016/j.micpath.2019.103748

Rekawiecki, R., Rutkowska, J. and Kotwica, J. (2012). Identification of optimal housekeeping genes for examination of gene expression in bovine corpus luteum. *Reproductive Biology*, *12*(4), 362–367. https://doi.org/10.1016/j.repbio.2012.10.010

Responsible Use of Medicines in Agriculture Alliance, R. (2015). *Responsible use of antimicrobials in cattle production*.

Rice, D. H., Besser, T. E. and Hancock, D. D. (1997). Epidemiology and virulence assessment of Salmonella dublin. *Veterinary Microbiology*, *56*(1–2), 111–124. https://doi.org/10.1016/S0378-1135(96)01352-1

Rice, P., Longden, L. and Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics*, *16*(6), 276–277. https://doi.org/10.1016/S0168-9525(00)02024-2

Richter-Dahlfors, A., Buchan, A. M. J. and Finlay, B. B. (1997). Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *Journal of Experimental Medicine*, *186*(4), 569–580. https://doi.org/10.1084/jem.186.4.569

Riordan, K. E. and Schneewind, O. (2008). YscU cleavage and the assembly of Yersinia type III secretion machine complexes. *Molecular Microbiology*, *68*(6), 1485. https://doi.org/10.1111/J.1365-2958.2008.06247.X

Robbins, J. R. and Bakardjiev, A. I. (2012). Pathogens and the placental fortress. *Current Opinion in Microbiology*, *15*(1), 36–43. https://doi.org/10.1016/j.mib.2011.11.006

Roblin, P., Dewitte, F., Villeret, V., Biondi, E. G. and Bompard, C. (2015). A Salmonella type three secretion effector/chaperone complex adopts a hexameric ring-like structure. *Journal of Bacteriology*, *197*(4), 688–698. https://doi.org/10.1128/JB.02294-14

Rolhion, N., Furniss, R. C. D., Grabe, G., Ryan, A., Liu, M., Matthews, S. A. and Holden, D. W. (2016). Inhibition of Nuclear Transport of NF-κB p65 by the Salmonella Type III Secretion System Effector SpvD. *PLoS Pathogens*, *12*(5), e1005653. https://doi.org/10.1371/JOURNAL.PPAT.1005653

Römling, U., Bian, Z., Hammar, M., Sierralta, W. D. and Normark, S. (1998). Curli Fibers Are Highly Conserved between Salmonella typhimurium and Escherichia coli with Respect to Operon Structure and Regulation. *Journal of Bacteriology*, *180*(3), 722. https://doi.org/10.1128/JB.180.3.722-731.1998

Rosbottom, A., Gibney, E. H., Guy, C. S., Kipar, A., Smith, R. F., Kaiser, P., Trees, A. J. and Williams, D. J. L. (2008). Upregulation of Cytokines Is Detected in the Placentas of Cattle Infected with Neospora caninum and Is More Marked Early in Gestation When Fetal Death Is Observed. *Infection and Immunity*, *76*(6), 2352. https://doi.org/10.1128/IAI.01780-06

Roy, M. F. and Malo, D. (2002). Genetic regulation of host responses to Salmonella infection in mice. *Genes and Immunity*, *3*(7), 381–393. https://doi.org/10.1038/sj.gene.6363924

RSPCA. (2018). RSPCA welfare standards for dairy cattle.

Ruiz-Albert, J., Yu, X. J., Beuzón, C. R., Blakey, A. N., Galyov, E. E. and Holden, D. W. (2002). Complementary activities of SseJ and SifA regulate dynamics of the Salmonella typhimurium vacuolar membrane. *Molecular Microbiology*, *44*(3), 645–661. https://doi.org/10.1046/J.1365-2958.2002.02912.X

Rupp, S., Aguilar-Bultet, L., Jagannathan, V., Guldimann, C., Pfarrer, C., Vidondo, B., Seuberlich, T., Frey, J., Oevermann, A., Drögemüller, C., Pfarrer, C., Vidondo, B., Seuberlich, T., Frey, J. and Oevermann, A. (2015). A naturally occurring prfA truncation in a Listeria monocytogenes field strain contributes to reduced replication and cell-to-cell spread. *Veterinary Microbiology*, *179*(1–2), 91–101. https://doi.org/10.1016/j.vetmic.2015.03.002

Rupp, S., Bartschi, M., Frey, J. and Oevermann, A. (2017). Hyperinvasiveness and increased intercellular spread of Listeria monocytogenes sequence type 1 are independent of listeriolysin S, internalin F and internalin J1. *Journal of Medical Microbiology*, *66*, 1053–1062. https://doi.org/10.1099/jmm.0.000529

Rydell-Törmänen, K., Uller, L. and Erjefält, J. S. (2006). Direct evidence of secondary necrosis of neutrophils during intense lung inflammation. *The European Respiratory Journal*, *28*(2), 268–274. https://doi.org/10.1183/09031936.06.00126905

Sabag-Daigle, A., Blunk, H. M., Gonzalez, J. F., Steidley, B. L., Boyaka, P. N. and Ahmer, B. M. M. (2016). Use of attenuated but metabolically competent Salmonella as a probiotic to prevent or treat Salmonella infection. *Infection and Immunity*, *84*(7), 2131–2140. https://doi.org/10.1128/IAI.00250-16

Sabbagh, S. C., Lepage, C., McClelland, M. and Daigle, F. (2012). Selection of Salmonella enterica Serovar Typhi Genes Involved during Interaction with Human Macrophages by Screening of a

Transposon Mutant Library. *PLOS ONE*, *7*(5), e36643. https://doi.org/10.1371/JOURNAL.PONE.0036643

Sabroe, I., Prince, L. R., Jones, E. C., Horsburgh, M. J., Foster, S. J., Vogel, S. N., Dower, S. K. and Whyte, M. K. B. (2003). Selective Roles for Toll-Like Receptor (TLR)2 and TLR4 in the Regulation of Neutrophil Activation and Life Span. *The Journal of Immunology*, *170*(10), 5268–5275. https://doi.org/10.4049/JIMMUNOL.170.10.5268

Saffarzadeh, M., Juenemann, C., Queisser, M. A., Lochnit, G., Barreto, G., Galuska, S. P., Lohmeyer, J. and Preissner, K. T. (2012). Neutrophil Extracellular Traps Directly Induce Epithelial and Endothelial Cell Death: A Predominant Role of Histones. *PLoS ONE*, *7*(2), 32366. https://doi.org/10.1371/JOURNAL.PONE.0032366

Saini, S., Pearl, J. A. and Rao, C. V. (2009). Role of FimW, FimY, and FimZ in regulating the expression of type I fimbriae in salmonella enterica serovar typhimurium. *Journal of Bacteriology*, *191*(9), 3003–3010. https://doi.org/10.1128/JB.01694-08

Salcedo, S. P. and Holden, D. W. (2003). SseG, a virulence protein that targets Salmonella to the Golgi network. *The EMBO Journal*, *22*(19), 5003. https://doi.org/10.1093/EMBOJ/CDG517

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). NY: Cold Spring Harbor Laboratory Press.

Sammin, D. J., Markey, B. K., Quinn, P. J., McElroy, M. C. and Bassett, H. F. (2006). Comparison of Fetal and Maternal Inflammatory Responses in the Ovine Placenta after Experimental Infection with Chlamydophila abortus. *Journal of Comparative Pathology*, *135*(2–3), 83–92. https://doi.org/10.1016/J.JCPA.2006.04.005

Sana, T. G., Flaugnatti, N., Lugo, K. A., Lam, L. H., Jacobson, A., Baylot, V., Durand, E., Journet, L., Cascales, E. and Monack, D. M. (2016). Salmonella Typhimurium utilizes a T6SS-mediated antibacterial weapon to establish in the host gut. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(34), E5044–E5051. https://doi.org/10.1073/PNAS.1608858113

Sana, T. G., Lugo, K. A. and Monack, D. M. (2017). T6SS: The bacterial "fight club" in the host gut. *PLOS Pathogens*, *13*(6), e1006325. https://doi.org/10.1371/JOURNAL.PPAT.1006325

Sayers, S., Li, L., Ong, E., Deng, S., Fu, G., Lin, Y., Yang, B., Zhang, S., Fa, Z., Zhao, B., Xiang, Z., Li, Y., Zhao, X. M., Olszewski, M. A., Chen, L. and He, Y. (2019). Victors: a web-based knowledge base of virulence factors in human and animal pathogens. *Nucleic Acids Research*, *47*(D1), D693–D700. https://doi.org/10.1093/NAR/GKY999

Schlafer, D. H., Fisher, P. J. and Davies, C. J. (2000). The bovine placenta before and after birth: placental development and function in health and disease. *Animal Reproduction Science*, *60–61*, 145–160. https://doi.org/10.1016/S0378-4320(00)00132-9

Schroll, C., Huang, K., Ahmed, S., Kristensen, B. M., Pors, S. E., Jelsbak, L., Lemire, S., Thomsen, L. E., Christensen, J. P., Jensen, P. R. and Olsen, J. E. (2019). The SPI-19 encoded type-six secretion-systems (T6SS) of Salmonella enterica serovars Gallinarum and Dublin play different roles during infection. *Veterinary Microbiology*, *230*, 23–31. https://doi.org/10.1016/J.VETMIC.2019.01.006

Selander, R. K., Smith, N. H., Beltran, P., Ferris, K. E., Kopecko, D. J., Rubin, F. A. and Reed Anny, W. (1992). Molecular Evolutionary Genetics of the Cattle-Adapted Serovar Salmonella dublin. *Journal of Bacteriology*, *174*(11), 3587–3592. https://doi.org/10.1128/jb.174.11.3587-3592.1992

Senger, P. L. (2012). *Pathways to Pregnancy and Parturition* (3rd ed.). Current Conceptions, INC.

Serezani, C. H., Chung, J., Ballinger, M. N., Moore, B. B., Aronoff, D. M. and Peters-Golden, M. (2012). Prostaglandin E2 Suppresses Bacterial Killing in Alveolar Macrophages by Inhibiting NADPH Oxidase. *American Journal of Respiratory Cell and Molecular Biology*, *37*(5), 562–570. https://doi.org/10.1165/RCMB.2007-0153OC

Seribelli, A. A., da Silva, P., Frazão, M. R., Kich, J. D., Allard, M. W. and Falcão, J. P. (2021). Phylogenetic relationship and genomic characterization of Salmonella Typhimurium strains isolated from swine in Brazil. *Infection, Genetics and Evolution*, *93*, 104977. https://doi.org/10.1016/J.MEEGID.2021.104977

Seth-Smith, H. M. B., Fookes, M. C., Okoro, C. K., Baker, S., Harris, S. R., Scott, P., Pickard, D., Quail, M. A., Churcher, C., Sanders, M., Harmse, J., Dougan, G., Parkhill, J. and Thomson, N. R. (2012). Structure, Diversity, and Mobility of the Salmonella Pathogenicity Island 7 Family of Integrative and Conjugative Elements within Enterobacteriaceae. *Journal of Bacteriology*, *194*(6), 1494. https://doi.org/10.1128/JB.06403-11

Shaik, S., Wu, X., Gimble, J. and Devireddy, R. (2018). Effects of Decade Long Freezing Storage on Adipose Derived Stem Cells Functionality. *Scientific Reports*, *8*(1), 8162. https://doi.org/10.1038/S41598-018-26546-7

Shea, J. E., Hensel, M., Gleeson, C. and Holden, D. W. (1996). Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(6), 2593. https://doi.org/10.1073/PNAS.93.6.2593

Shinomiya, S., Naraba, H., Ueno, A., Utsunomiya, I., Maruyama, T., Ohuchida, S., Ushikubi, F., Yuki, K., Narumiya, S., Sugimoto, Y.,

Ichikawa, A. and Oh-ishi, S. (2001). Regulation of TNFalpha and interleukin-10 production by prostaglandins I(2) and E(2): studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. *Biochemical Pharmacology*, *61*(9), 1153–1160. https://doi.org/10.1016/S0006-2952(01)00586-X

Shishikura, K., Horiuchi, T., Sakata, N., Trinh, D. A., Shirakawa, R., Kimura, T., Asada, Y. and Horiuchi, H. (2016). Prostaglandin E2 inhibits neutrophil extracellular trap formation through production of cyclic AMP. *British Journal of Pharmacology*, *173*(2), 319–331. https://doi.org/10.1111/BPH.13373

Silva, A. P. C., Costa, É. A., Macêdo, A. A., Martins, T. da M., Borges, Á. M., Paixão, T. A. and Santos, R. L. (2012). Transcription of pattern recognition receptors and abortive agents induced chemokines in the bovine pregnant uterus. *Veterinary Immunology and Immunopathology*, *145*(1–2), 248–256. https://doi.org/10.1016/j.vetimm.2011.11.007

Silva, M., Machado, M. P., Silva, D. N., Rossi, M., Moran-Gilad, J., Santos, S., Ramirez, M. and Ao Andr E Carriço, J. (2018). chewBBACA: A complete suite for gene-by-gene schema creation and strain identification. *Microbial Genomics*, *4*(3), e000166. https://doi.org/10.1099/mgen.0.000166

Silverman, J. M., Brunet, Y. R., Cascales, E. and Mougous, J. D. (2012). Structure and Regulation of the Type VI Secretion System. *Annual Review of Microbiology*, *66*, 453. https://doi.org/10.1146/ANNUREV-MICRO-121809-151619

Singh, V., Yeoh, B. S., Chassaing, B., Zhang, B., Saha, P., Xiao, X., Awasthi, D., Shashidharamurthy, R., Dikshit, M., Gewirtz, A. and Vijay-Kumar, M. (2016). Microbiota-Inducible Innate Immune Siderophore Binding Protein Lipocalin 2 Is Critical for Intestinal Homeostasis. *Cellular and Molecular Gastroenterology and*

Hepatology, *2*(4), 482–498. https://doi.org/10.1016/J.JCMGH.2016.03.007

Smith, M. G. (1977). Transfer of R factors from Escherichia coli to salmonellas in the rumen of sheep. *Journal of Medical Microbiology*, *10*(1), 29–35. https://doi.org/10.1099/00222615-10-1-29

Smith, R. L., Kaczmarek, M. T., Kucharski, L. M. and Maguire, M. E. (1998). Magnesium transport in Salmonella typhimurium: Regulation of mgtA and mgtCB during invasion of epithelial and macrophage cells. *Microbiology*, *144*(7), 1835–1843. https://doi.org/10.1099/00221287-144-7-1835

Somerwill, R. (2014). *The Effects of Schmallenberg Virus on Interferon Tau Expression by Bovine Placental Cells*. University of Nottingham.

Sorg, I., Wagner, S., Amstutz, M., Müller, S. A., Broz, P., Lussi, Y., Engel, A. and Cornelis, G. R. (2007). YscU recognizes translocators as export substrates of the Yersinia injectisome. *The EMBO Journal*, *26*(12), 3015. https://doi.org/10.1038/SJ.EMBOJ.7601731

Sousa, R. O., Cariaco, Y., Almeida, M. P. O., Nascimento, L. A. C., Coutinho, L. B., Ferreira-Júnior, A. A., Briceño, M. P. P., Venâncio, M. de F. A., Oliveira, M. C., Miranda, N. C., Pajuaba, A. C. A. M., Ferro, E. A. V., Filice, L. de S. C. and Silva, N. M. (2021). The imbalance of TNF and IL-6 levels and FOXP3 expression at the maternal-fetal interface is involved in adverse pregnancy outcomes in a susceptible murine model of congenital toxoplasmosis. *Cytokine*, *143*, 155517. https://doi.org/10.1016/J.CYTO.2021.155517

Spöring, I., Felgner, S., Preuße, M., Eckweiler, D., Rohde, M., Häussler, S., Weiss, S. and Erhardt, M. (2018). Regulation of flagellum biosynthesis in response to cell envelope stress in

salmonella enterica serovar Typhimurium. *MBio*, *9*(3), e00736. https://doi.org/10.1128/MBIO.00736-17

Srednik, M. E., Lantz, K., Hicks, J. A., Morningstar-Shaw, B. R., Mackie, T. A. and Schlater, L. K. (2021). Antimicrobial resistance and genomic characterization of Salmonella Dublin isolates in cattle from the United States. *PLoS ONE*, *16*(9), e0249617. https://doi.org/10.1371/JOURNAL.PONE.0249617

Sreekantapuram, S., Berens, C., Barth, S. A., Methner, U. and Berndt, A. (2021). Interaction of Salmonella Gallinarum and Salmonella Enteritidis with peripheral leucocytes of hens with different laying performance. *Veterinary Research*, *52*(1), 123. https://doi.org/10.1186/S13567-021-00994-Y

Sreekantapuram, S., Lehnert, T., Praußе, M. T. E., Berndt, A., Berens, C., Figge, M. T. and Jacobsen, I. D. (2020). Dynamic Interplay of Host and Pathogens in an Avian Whole-Blood Model. *Frontiers in Immunology*, *11*, 500. https://doi.org/10.3389/FIMMU.2020.00500

Stanaway, J. D., Parisi, A., Sarkar, K., … Crump, J. A. (2019). The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*, *19*(12), 1312–1324. https://doi.org/10.1016/S1473-3099(19)30418-9

Stanaway, J. D., Reiner, R. C., Blacker, B. F., … Hay, S. I. (2019). The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*, *19*(4), 369–381. https://doi.org/10.1016/S1473-3099(18)30685-6

Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M. J., Pickard, D. J., Barthel, M., Westendorf, A. M., Krogfelt, K. A., Walker, A. W., Ackermann, M., Dobrindt, U., Thomson, N. R. and Hardt, W. D. (2012). Gut inflammation can boost horizontal gene transfer

between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(4), 1269–1274. https://doi.org/10.1073/PNAS.1113246109

Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., Lee, J. C. and Dubinett, S. M. (2000). Specific Inhibition of Cyclooxygenase 2 Restores Antitumor Reactivity by Altering the Balance of IL-10 and IL-12 Synthesis. *The Journal of Immunology*, *164*(1), 361–370. https://doi.org/10.4049/JIMMUNOL.164.1.361

Takeuchi, A. (1967). Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by Salmonella typhimurium. *The American Journal of Pathology*, *50*(1), 109.

Talbott, H., Delaney, A., Zhang, P., Yu, Y., Cushman, R. A., Cupp, A. S., Hou, X. and Davis, J. S. (2014). Effects of IL8 and Immune Cells on the Regulation of Luteal Progesterone Secretion. *Reproduction*, *148*(1), 21–31. https://doi.org/10.1530/REP-13-0602

Tangri, S. and Raghupathy, R. (1993). Expression of cytokines in placentas of mice undergoing immunologically mediated spontaneous fetal resorptions. *Biology of Reproduction*, *49*(4), 850–856. https://doi.org/10.1095/BIOLREPROD49.4.850

Tena, G. N., Young, D. B., Eley, B., Henderson, H., Nicol, M. P., Levin, M. and Kampman, B. (2003). Failure to Control Growth of Mycobacteria in Blood from Children Infected with Human Immunodeficiency Virus and Its Relationship to T Cell Function. *The Journal of Infectious Diseases*, *187*(10), 1544–1551. https://doi.org/10.1086/374799

Tili, E., Michaille, J.-J., Cimino, A., Costinean, S., Dumitru, C. D., Adair, B., Fabbri, M., Alder, H., Liu, C. G., Calin, G. A. and Croce,

C. M. (2007). Modulation of miR-155 and miR-125b Levels following Lipopolysaccharide/TNF-α Stimulation and Their Possible Roles in Regulating the Response to Endotoxin Shock. *The Journal of Immunology*, *179*(8), 5082–5089. https://doi.org/10.4049/JIMMUNOL.179.8.5082

Tinker, J. K., Hancox, L. S. and Clegg, S. (2001). FimW Is a Negative Regulator Affecting Type 1 Fimbrial Expression in Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology*, *183*(2), 435. https://doi.org/10.1128/JB.183.2.435-442.2001

Toledo-Arana, A., Dussurget, O., Nikitas, G., … Cossart, P. (2009). The Listeria transcriptional landscape from saprophytism to virulence. *Nature*, *459*(7249), 950–956. https://doi.org/10.1038/nature08080

Troxell, B., Fink, R. C., Porwollik, S., McClelland, M. and Hassan, H. M. (2011). The fur regulon in anaerobically grown Salmonella enterica sv. Typhimurium: Identification of new fur targets. *BMC Microbiology*, *11*(1), 1–19. https://doi.org/10.1186/1471-2180-11-236

Tükel, Ç., Nishimori, J. H., Wilson, R. P., Winter, M. G., Keestra, A. M., Van Putten, J. P. M. and Bäumler, A. J. (2010). Toll-like receptors 1 and 2 cooperatively mediate immune responses to curli, a common amyloid from enterobacterial biofilms. *Cellular Microbiology*, *12*(10), 1495–1505. https://doi.org/10.1111/J.1462-5822.2010.01485.X

Ung, A., Baidjoe, A. Y., Van Cauteren, D., Fawal, N., Fabre, L., Guerrisi, C., Danis, K., Morand, A., Donguy, M.-P., Lucas, E., Rossignol, L., Lefèvre, S., Vignaud, M.-L., Cadel-Six, S., Lailler, R., Jourdan-Da Silva, N. and Le Hello, S. (2019). Disentangling a complex nationwide Salmonella Dublin outbreak associated with raw-milk cheese consumption, France, 2015 to 2016. *Eurosurveillance*, *24*(3), 1700703. https://doi.org/10.2807/1560-7917.ES.2019.24.3.1700703

Usui, M., Nagai, H., Hiki, M., Tamura, Y. and Asai, T. (2013). Effect of antimicrobial exposure on AcrAB expression in Salmonella enterica subspecies enterica serovar Choleraesuis. *Frontiers in Microbiology*, *4*, 53. https://doi.org/10.3389/FMICB.2013.00053

Vaessen, M. A., Veling, J., Frankena, K., Graat, E. A. M. and Klunder, T. (1998). Risk factors for *salmonella dublin* infection on dairy farms. *Veterinary Quarterly*, *20*(3), 97–99. https://doi.org/10.1080/01652176.1998.9694848

Valdivia, R. H. and Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science*, *277*(5334), 2007–2011. https://doi.org/10.1126/SCIENCE.277.5334.2007

van der Pouw Kraan, T. C. T. M., Boeije, L. C. M., Smeenk, R. J. T., Wijdenes, J. and Aarden, L. A. (1995). Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *The Journal of Experimental Medicine*, *181*(2), 775–779. https://doi.org/10.1084/JEM.181.2.775

Van Engelen, E., De Groot, M. W., Breeveld-Dwarkasing, V. N. A., Everts, M. E., Van Der Weyden, G. C., Taverne, M. A. M. and Rutten, V. P. M. G. (2009). Cervical Ripening and Parturition in Cows are Driven by a Cascade of Pro-Inflammatory Cytokines. *Reproduction in Domestic Animals*, *44*(5), 834–841. https://doi.org/10.1111/J.1439-0531.2008.01096.X

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), 1–12. https://doi.org/10.1186/GB-2002-3-7-RESEARCH0034

Vazquez-Terres, A., Jones-Carson, J., Bäumler, A. J., Falkow, S., Valdivia, R., Brown, W., Lo, M., Berggren, R., Parks, W. T. and Fang, F. C. (1999). Extraintestinal dissemination of Salmonella

by CD18-expressing phagocytes. *Nature*, *401*(6755), 804–808. https://doi.org/10.1038/44593

Vázquez-Torres, A., Fantuzzi, G., Edwards, C. K., Dinarello, C. A. and Fang, F. C. (2001). Defective localization of the NADPH phagocyte oxidase to Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(5), 2561–2565. https://doi.org/10.1073/PNAS.041618998

Vazquez-Torres, A., Vallance, B. A., Bergman, M. A., Finlay, B. B., Cookson, B. T., Jones-Carson, J. and Fang, F. C. (2004). Toll-Like Receptor 4 Dependence of Innate and Adaptive Immunity to Salmonella: Importance of the Kupffer Cell Network. *The Journal of Immunology*, *172*(10), 6202–6208. https://doi.org/10.4049/JIMMUNOL.172.10.6202

Velge, P., Wiedemann, A., Rosselin, M., Abed, N., Boumart, Z., Chaussé, A. M., Gré Pinet, O., Namdari, F., Roche, S. M., Rossignol, A. and Virlogeux-Payant, I. (2012). Multiplicity of Salmonella entry mechanisms, a new paradigm for Salmonella pathogenesis. *MicrobiologyOpen*, *1*(3), 243–258. https://doi.org/10.1002/mbo3.28

Veterinary Medicines Directorate. (2016a). *UK Veterinary Antibiotic Resistance and Sales Surveillance Report*.

Veterinary Medicines Directorate. (2016b). *UK Veterinary Antibiotic Resistance and Sales Surveillance Report Supplementary Material*.

Veterinary Medicines Directorate. (2017). *UK Veterinary Antibiotic Resistance and Sales Surveillance Report 2017*.

Veterinary Medicines Directorate. (2018). *UK Veterinary Antibiotic Resistance and Sales Surveillance Report 2018*.

Veterinary Medicines Directorate. (2019). UK One Health Report - Joint report on antibiotic use and antibiotic resistance, 2013–2017.

Veterinary Medicines Directorate. (2020). *UK Veterinary Antibiotic Resistance and Sales Surveillance Report Supplementary Material*.

Vilela, F. P., dos Prazeres Rodrigues, D., Costa, R. G., Casas, M. R. T., Falcão, J. P. and Campioni, F. (2020). High similarity and high frequency of virulence genes among Salmonella Dublin strains isolated over a 33-year period in Brazil. *Brazilian Journal of Microbiology*, *51*(2), 497–509. https://doi.org/10.1007/s42770-019-00156-5

Vohra, P., Bugarel, M., Turner, F., Loneragan, G. H., Hope, J. C., Hopkins, J. and Stevens, M. P. (2018). Quantifying the survival of multiple Salmonella enterica serovars in vivo via massively parallel whole-genome sequencing to predict zoonotic risk. *Applied and Environmental Microbiology*, *84*(4), e02262. https://doi.org/10.1128/AEM.02262-17

Vohra, P., Vrettou, C., Hope, J. C., Hopkins, J. and Stevens, M. P. (2019). Nature and consequences of interactions between Salmonella enterica serovar Dublin and host cells in cattle. *Veterinary Research*, *50*(1), 99. https://doi.org/10.1186/S13567-019-0720-5

Waldner, L., MacKenzie, K., Köster, W. and White, A. (2012). From Exit to Entry: Long-term Survival and Transmission of Salmonella. *Pathogens*, *1*(2), 128–155. https://doi.org/10.3390/pathogens1020128

Walker, W. and Rotondo, D. (2004). Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-γ synthesis. *Immunology*, *111*(3), 298. https://doi.org/10.1111/J.1365-2567.2004.01810.X

Wang, F., Deng, L., Huang, F., Wang, Z., Lu, Q. and Xu, C. (2020). Flagellar Motility Is Critical for Salmonella enterica Serovar Typhimurium Biofilm Development. *Frontiers in Microbiology*, *11*, 1695. https://doi.org/10.3389/FMICB.2020.01695

Wang, Z., Zhang, Y., Xu, H., Chu, C., Wang, J., Jiao, X. and Li, Q. (2022). Whole-genome sequencing analysis reveals pig as the main reservoir for persistent evolution of Salmonella enterica serovar Rissen causing human salmonellosis. *Food Research International*, *154*, 111007. https://doi.org/10.1016/J.FOODRES.2022.111007

Warburg, O. and Christian, W. (1942). Isolation and crystallisation of enolase. *Biochem Z*, *310*, 384–421.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. and Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189–1191. https://doi.org/10.1093/BIOINFORMATICS/BTP033

Waterkotte, B., Hambruch, N., Döring, B., Geyer, J., Tinneberg, H.-R. and Pfarrer, C. (2011). P-glycoprotein is functionally expressed in the placenta-derived bovine caruncular epithelial cell line 1 (BCEC-1). *Placenta*, *32*, 146–152. https://doi.org/10.1016/j.placenta.2010.11.009

Watson, P. R., Gautier, A. V., Paulin, S. M., Bland, A. P., Jones, P. W. and Wallis, T. S. (2000). Salmonella enterica serovars Typhimurium and Dublin can lyse macrophages by a mechanism distinct from apoptosis. *Infection and Immunity*, *68*(6), 3744–3747. https://doi.org/10.1128/IAI.68.6.3744-3747.2000

Wattam, A. R., Abraham, D., Dalay, O., … Sobral, B. W. (2014). PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Research*, *42*, D581–D591. https://doi.org/10.1093/NAR/GKT1099

Webber, M. A., Bailey, A. M., Blair, J. M. A., Morgan, E., Stevens, M. P., Hinton, J. C. D., Ivens, A., Wain, J. and Piddock, L. J. V. (2009). The global consequence of disruption of the AcrAB-TolC efflux pump in Salmonella enterica includes reduced expression of SPI-1 and other attributes required to infect the host. *Journal of Bacteriology*, *191*(13), 4276–4285. https://doi.org/10.1128/JB.00363-09

Weening, E. H., Barker, J. D., Laarakker, M. C., Humphries, A. D., Tsolis, R. M. and Bäumler, A. J. (2005). The Salmonella enterica Serotype Typhimurium lpf, bcf, stb, stc, std, and sth Fimbrial Operons Are Required for Intestinal Persistence in Mice, *73*(6), 3358–3366. https://doi.org/10.1128/IAI.73.6.3358-3366.2005

Wegmann, T. G., Lin, H., Guilbert, L. and Mosmann, T. R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunology Today*, *14*(7), 353–356. https://doi.org/10.1016/0167-5699(93)90235-D

Wellawa, D. H., Lam, P.-K. S., White, A. P., Gomis, S., Allan, B. and Köster, W. (2022). High Affinity Iron Acquisition Systems Facilitate but Are Not Essential for Colonization of Chickens by Salmonella Enteritidis. *Frontiers in Microbiology*, *13*, 280. https://doi.org/10.3389/FMICB.2022.824052

Weston, J. F., Murray, R., Borsberry, S., Cutler, K. and Murphy, A. (2012). Clinical Forum - Investigation of Bovine Abortion. *Livestock*, *17*(5), 15–24. https://doi.org/10.1111/j.2044-3870.2012.00135.x

Wheeler, N. E., Gardner, P. P. and Barquist, L. (2018). Machine learning identifies signatures of host adaptation in the bacterial pathogen Salmonella enterica. *PLOS Genetics*, *14*(5), e1007333. https://doi.org/10.1371/JOURNAL.PGEN.1007333

Wheelhouse, N. and Dagleish, M. (2014). Diagnosing the causes of ruminant abortion: Where are we now? *The Veterinary Journal*, *201*(3), 243–244. https://doi.org/10.1016/J.TVJL.2014.05.042

Wheelhouse, N., Wattegedera, S., Stanton, J., Maley, S., Watson, D., Jepson, C., Deane, D., Buxton, D., Longbottom, D., Baszler, T. and Entrican, G. (2009). Ovine trophoblast is a primary source of TNFα during Chlamydophila abortus infection. *Journal of Reproductive Immunology*, *80*(1–2), 49–56. https://doi.org/10.1016/J.JRI.2008.12.003

White, D. G., Goldman, J. D., Demple, B. and Levy, S. B. (1997). Role of the acrAB locus in organic solvent tolerance mediated by expression of marA, soxS, or robA in Escherichia coli. *Journal of Bacteriology*, *179*(19), 6122. https://doi.org/10.1128/JB.179.19.6122-6126.1997

Wilson, J. S., Hazel, S. M., Williams, N. J., Phiri, A., French, N. P. and Hart, C. A. (2003). Nontyphoidal Salmonellae in United Kingdom Badgers : Prevalence and Spatial Distribution. *Applied and Environmental Microbiology*, *69*(7), 4312–4315. https://doi.org/10.1128/AEM.69.7.4312

Wooding, P. and Burton, G. (2008). *Synepitheliochorial Placentation: Ruminants (Ewe and Cow). In: Comparative Placentation*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-78797-6

World Health Organisation. (2015). Global Action Plan on Antimicrobial Resistance.

World Health Organisation. (2017). *WHO Guidelines on use of medically important antimicrobials in food-producing animals*.

World Health Organisation. (2018). *Critically Important Antimicrobials for Human Medicine - 6th Revision 2018 - Ranking*

of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use.

Wu, H., Jones, R. M. and Neish, A. S. (2012). The Salmonella effector AvrA mediates bacterial intracellular survival during infection in vivo. *Cellular Microbiology*, *14*(1), 28–39. https://doi.org/10.1111/j.1462-5822.2011.01694.x

Xiang, Y., Wu, F., Chai, Y., Xu, X., Yang, L., Tian, S., Zhang, H., Li, Y., Yang, C., Liu, H., Qiu, S., Song, H. and Sun, Y. (2020). A new plasmid carrying mphA causes prevalence of azithromycin resistance in enterotoxigenic Escherichia coli serogroup O6. *BMC Microbiology*, *20*(1), 1–9. https://doi.org/10.1186/S12866-020-01927-Z

Yan, S., Liu, X., Li, C., Jiang, Z., Li, D. and Zhu, L. (2022). Genomic virulence genes profile analysis of Salmonella enterica isolates from animal and human in China from 2004 to 2019. *Microbial Pathogenesis*, *173*, 105808. https://doi.org/10.1016/J.MICPATH.2022.105808

Yim, L., Sasías, S., Martínez, A., Betancor, L., Estevez, V., Scavone, P., Bielli, A., Sirok, A. and Chabalgoity, J. A. (2014). Repression of flagella is a common trait in field isolates of Salmonella enterica serovar Dublin and is associated with invasive human infections. *Infection and Immunity*, *82*(4), 1465–1476. https://doi.org/10.1128/IAI.01336-13

Yu, X.-J., Grabe, G. J., Liu, M., Mota, L. J. and Holden, D. W. (2018). SsaV Interacts with SsaL to Control the Translocon-to-Effector Switch in the Salmonella SPI-2 Type Three Secretion System. *MBio*, *9*(5), e01149. https://doi.org/10.1128/mBio.01149-18

Yue, M., Rankin, S. C., Blanchet, R. T., Nulton, J. D., Edwards, R. A. and Schifferli, D. M. (2012). Diversification of the Salmonella Fimbriae: A Model of Macro- and Microevolution. *PLoS ONE*, *7*(6), e38596. https://doi.org/10.1371/JOURNAL.PONE.0038596

Zeinzinger, J., Pietzka, A. T., Stöger, A., Kornschober, C., Kunert, R., Allerberger, F., Mach, R. and Ruppitsch, W. (2012). One-Step Triplex High-Resolution Melting Analysis for Rapid Identification and Simultaneous Subtyping of Frequently Isolated Salmonella Serovars. *Applied and Environmental Microbiology*, *78*(9), 3352. https://doi.org/10.1128/AEM.07668-11

Zhang, H., Wu, Z. min, Yang, Y. ping, Shaukat, A., Yang, J., Guo, Y. fang, Zhang, T., Zhu, X. ying, Qiu, J. xia, Deng, G. zhen and Shi, D. mei. (2019). Catalpol ameliorates LPS-induced endometritis by inhibiting inflammation and TLR4/NF-κB signaling. *Journal of Zhejiang University. Science. B*, *20*(10), 816. https://doi.org/10.1631/JZUS.B1900071

Zhang, S., Santos, R. L., Tsolis, R. M., Stender, S., Hardt, W., Bäumler, A. J. and Adams, L. G. (2002). The Salmonella enterica Serotype Typhimurium Effector Proteins SipA , SopA , SopB , SopD , and SopE2 Act in Concert To Induce Diarrhea in Calves. *Infection and Immunity*, *70*(7), 3843–3855. https://doi.org/10.1128/IAI.70.7.3843

Zuo, L., Zhou, L., Wu, C., Wang, Y., Li, Y., Huang, R. and Wu, S. (2020). Salmonella spvC Gene Inhibits Pyroptosis and Intestinal Inflammation to Aggravate Systemic Infection in Mice. *Frontiers in Microbiology*, *11*, 3135. https://doi.org/10.3389/FMICB.2020.562491

Chapter 9 - Appendix

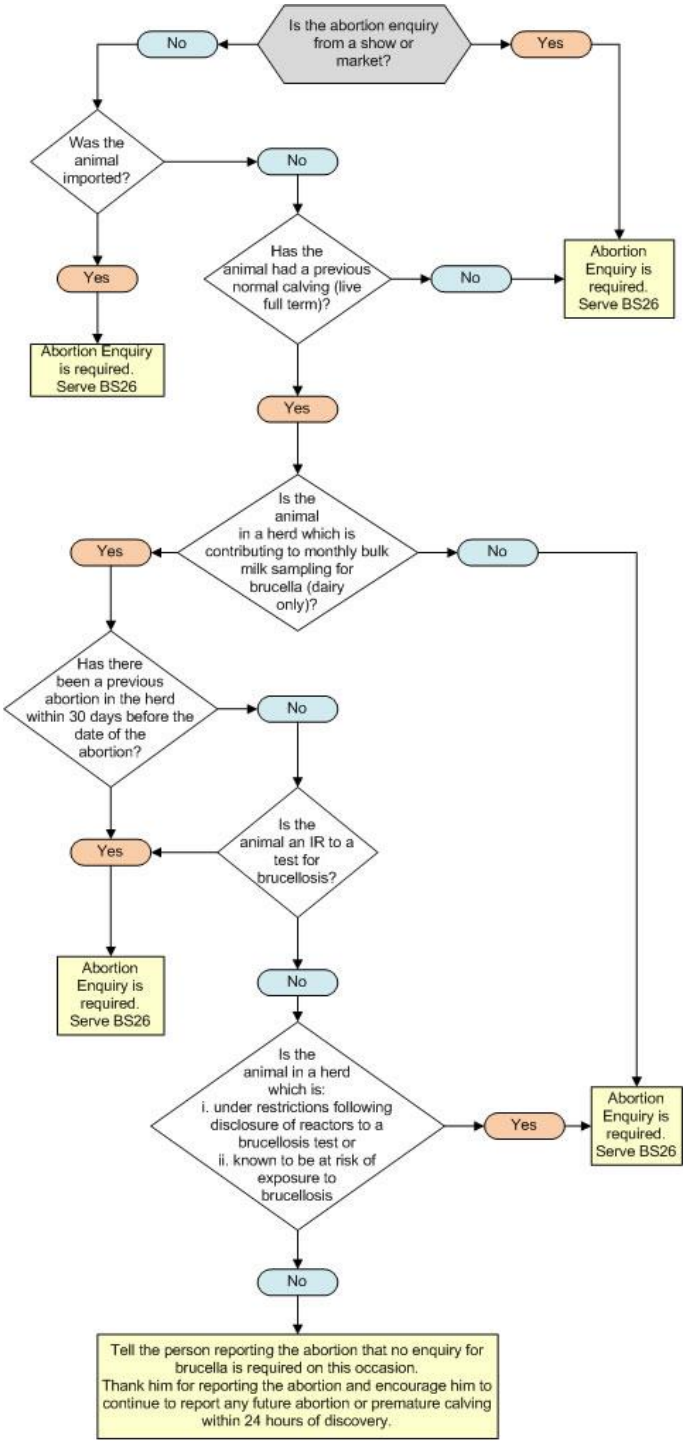


Figure 9.1 – Decision tree used by APHA in the process of abortion enquiries. All bovine abortions in the UK must be reported to the Animal and Plant Health Agency (APHA) who use this decision tree to inform the best course of action - either an abortion enquiry is required and samples need to be taken for testing, or the risk of brucella is low and therefore no enquiry is required (APHA, 2019a).

Table 9.1 - Anatomical locations from which *S.* Dublin has reportedly been isolated in the literature.

Organ/region *S.* Dublin isolated from	Authors and papers	Age of animal	Method of bacterial isolation
Faeces Rectum	Gitter et al, 1978	Calf	Direct plating onto deoxycholate-citrate agar
Liver Lungs Ovaries Placentomes/placenta Uterine wall	Hall and Jones, 1977	Adult cows	Samples from animals taken, spread onto modified brilliant green agar.
Prescapular lymph nodes	Hall and Jones, 1979	Calf	Enrichment media, Rappaport broth and selenite brilliant green broth
Cotyledonary fluid (fetal) Pleural fluid (fetal)		Foetus (aborted)	
Stomach (fetal) Abomasal wall Caecal wall Colon wall Mesenteric lymph nodes Retropharyngeal lymph nodes		Foetus (born alive)	
		Heifer	
Faeces Milk Placentomes/placenta Vaginal mucous	Hinton 1974	Heifer	Swabbed and cultured on MacConkey agar, then onto desoxycholate citrate agar
Abomasal contents Bile Duodenal contents Duodenal wall Gallbladder Heart Hepatic lymph nodes Kidney Liver Lungs Mesenteric lymph nodes Prefemoral/percural lymph nodes Prescapular lymph nodes Rumen contents Salivary gland Spleen Suprapharyngeal lymph nodes	Nazer and Osborne, 1977	Calves	Enrichment media, Rappaport broth and selenite brilliant green broth

Tonsil Urine			
Caecal contents Caecal mucosa Jejunal contents Jejunal lymph nodes Jejunal mucosa Liver Spleen	Steinbach et al, 1996	Calves	Samples taken, homogenised and grown on deoxycholate-citrate agar plates.
Caecal lymph nodes Distal ileum Mesenteric lymph nodes Popliteal lymph nodes Prefemoral/percural lymph nodes Prescapular lymph nodes	Vohra et al, 2017	Calves	Samples from animals taken, homogenised, bacteria grown on nutrient agar, lysed and DNA extracted and sequenced.

Age of the animal varies between studies, so have been grouped into fetal, calf, heifer and adult cow. Deoxycholate-citrate, Modified brilliant green agar, Selenite brilliant green and Rappaport broth are all used for selectively growing *Salmonella* species.

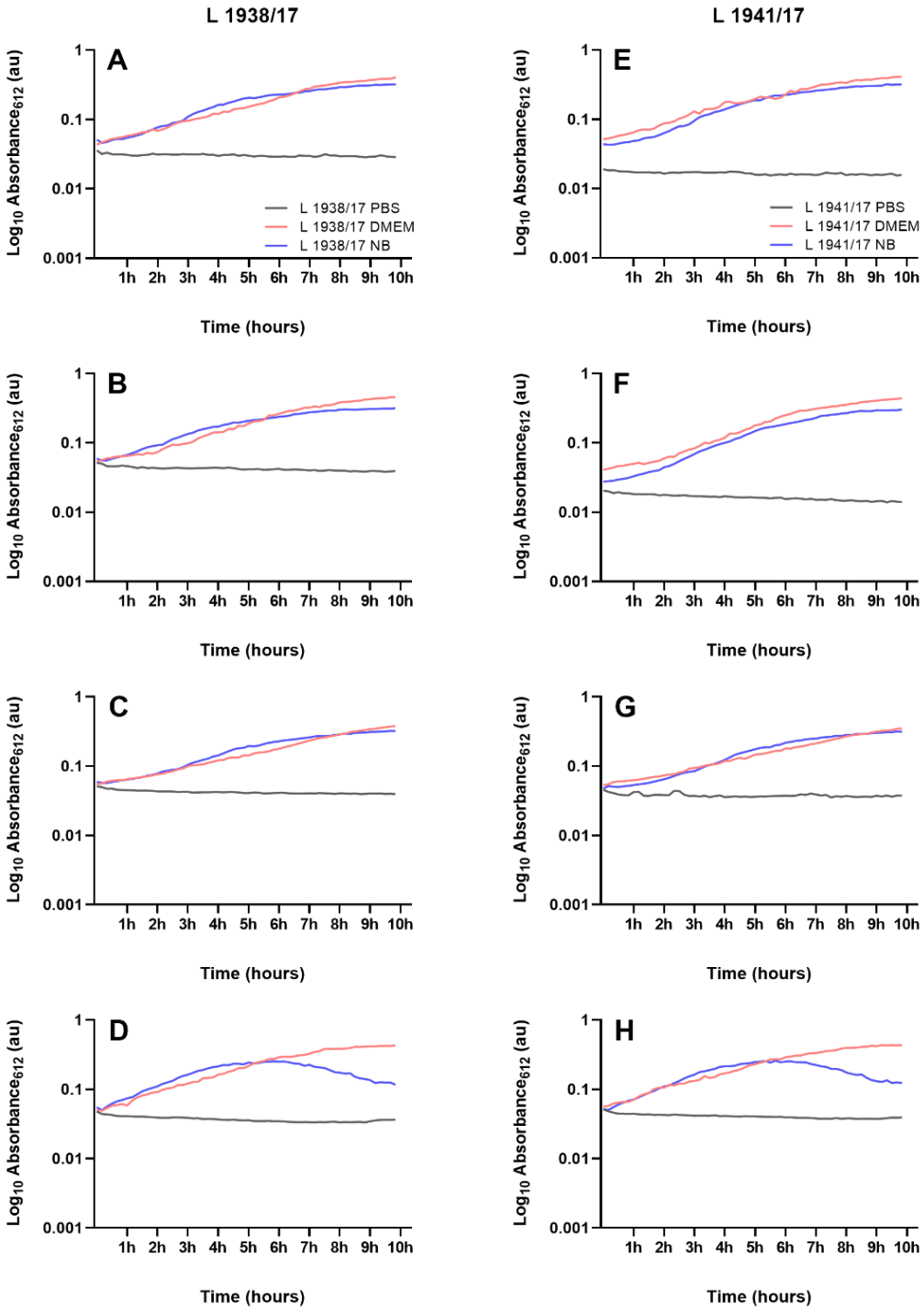


Figure 9.2 – Growth of *S.* Dublin isolates L 1938/17 (A-D) and L 1941/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

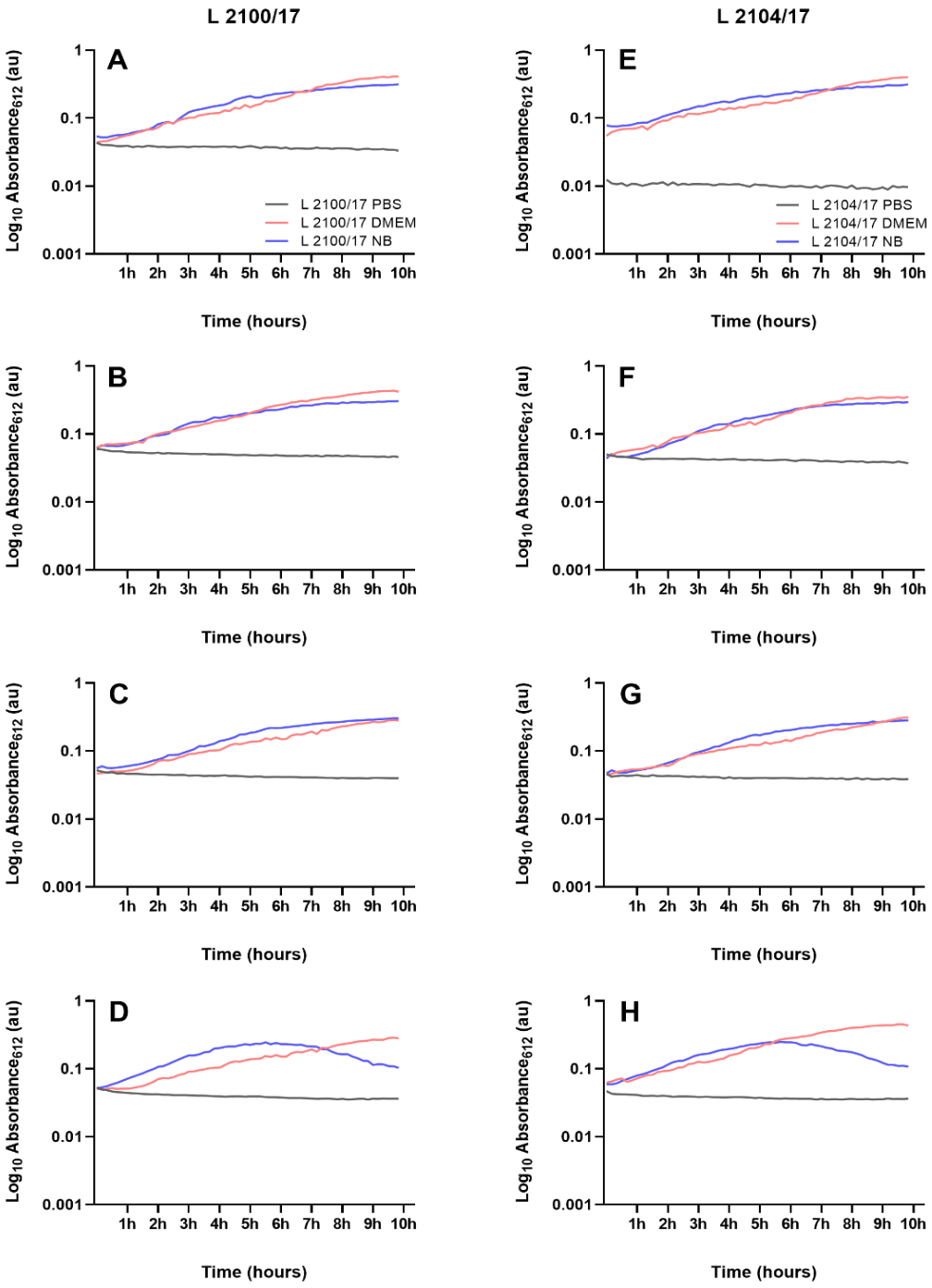


Figure 9.3 - Growth of S. Dublin isolates L 2100/17 (A-D) and L 2104/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

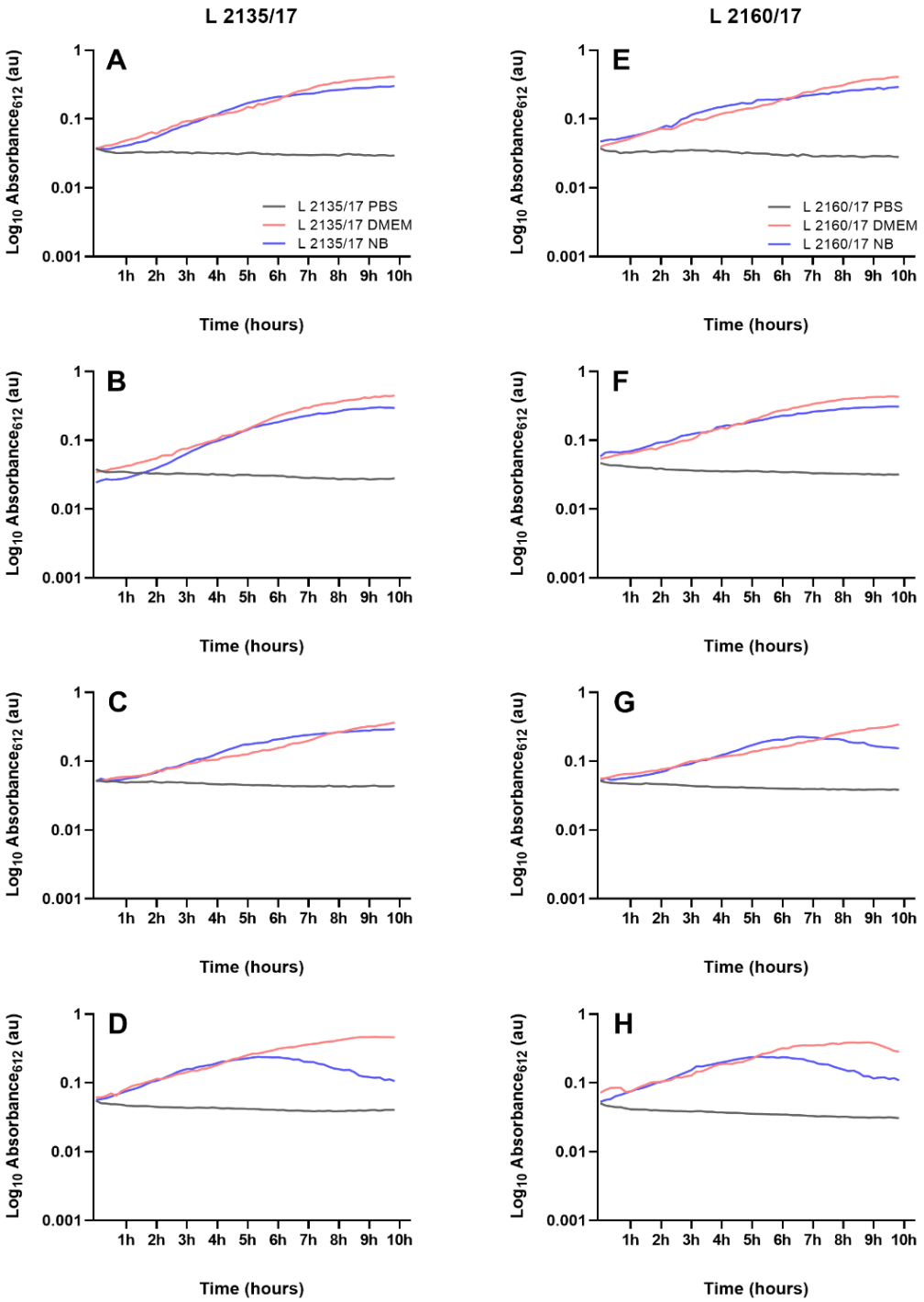


Figure 9.4 - Growth of S. Dublin isolates L 2135/17 (A-D) and L 2160/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

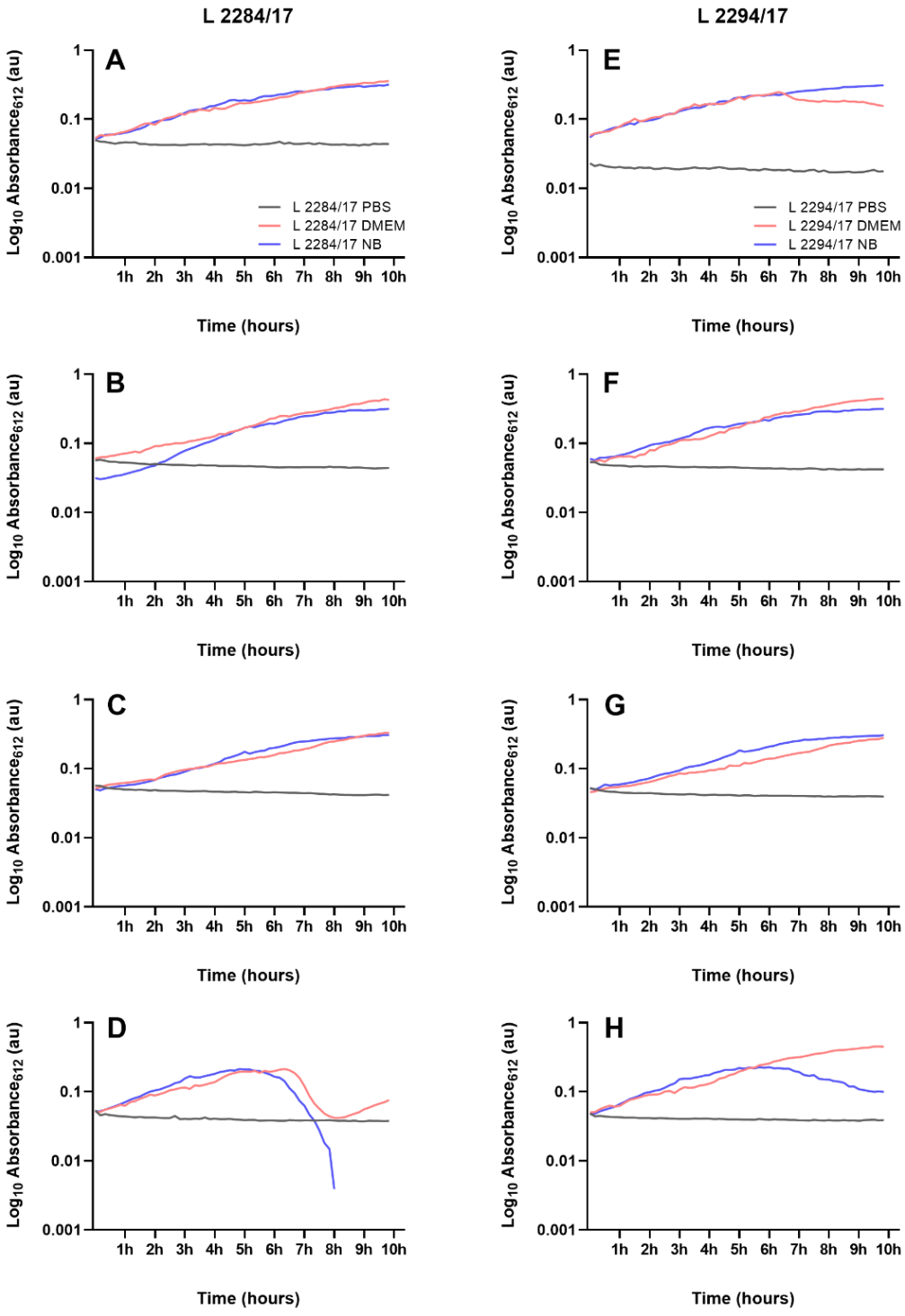


Figure 9.5 - Growth of S. Dublin isolates L 2284/17 (A-D) and L 2294/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

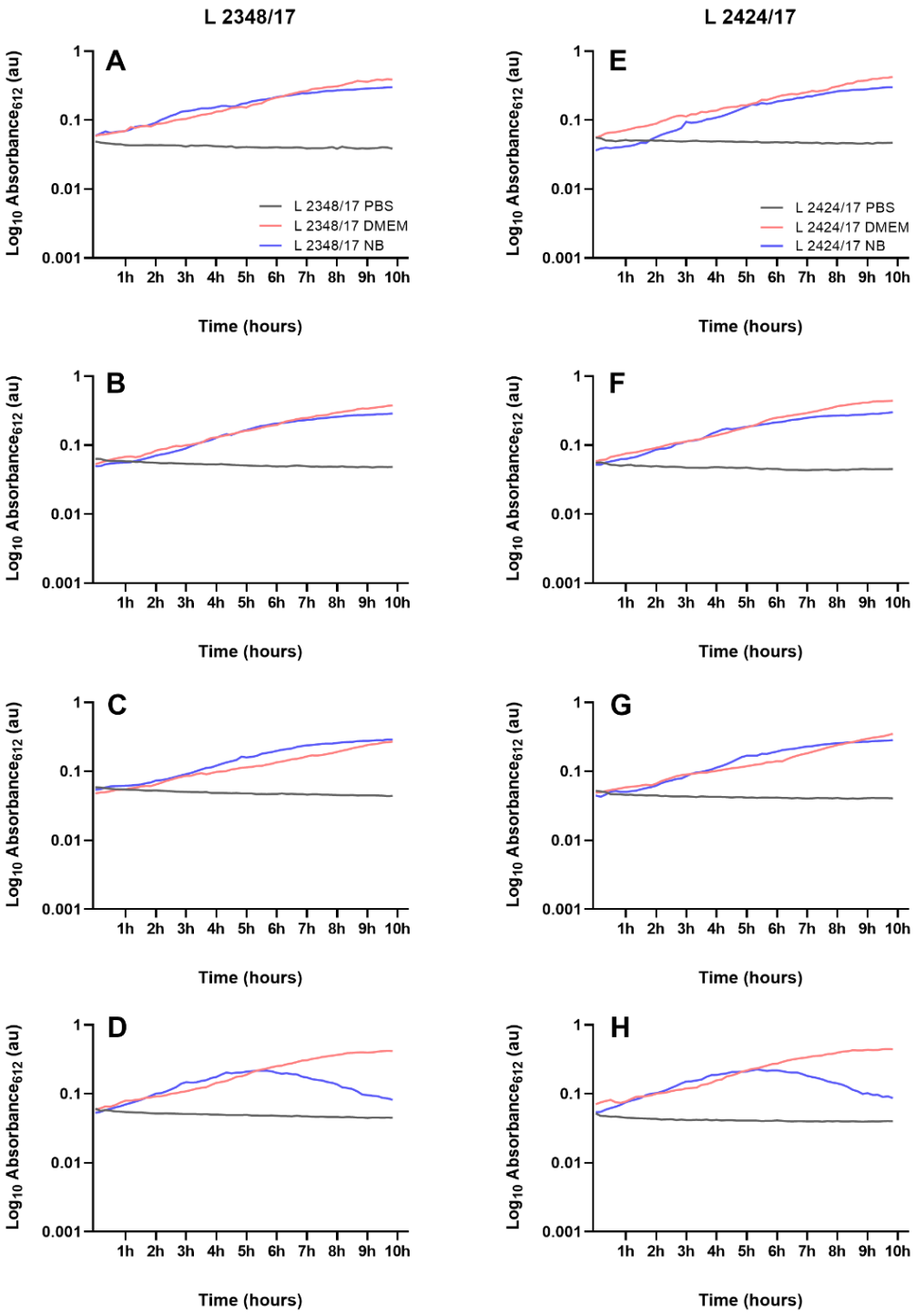


Figure 9.6 - Growth of S. Dublin isolates L 2348/17 (A-D) and L 2424/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

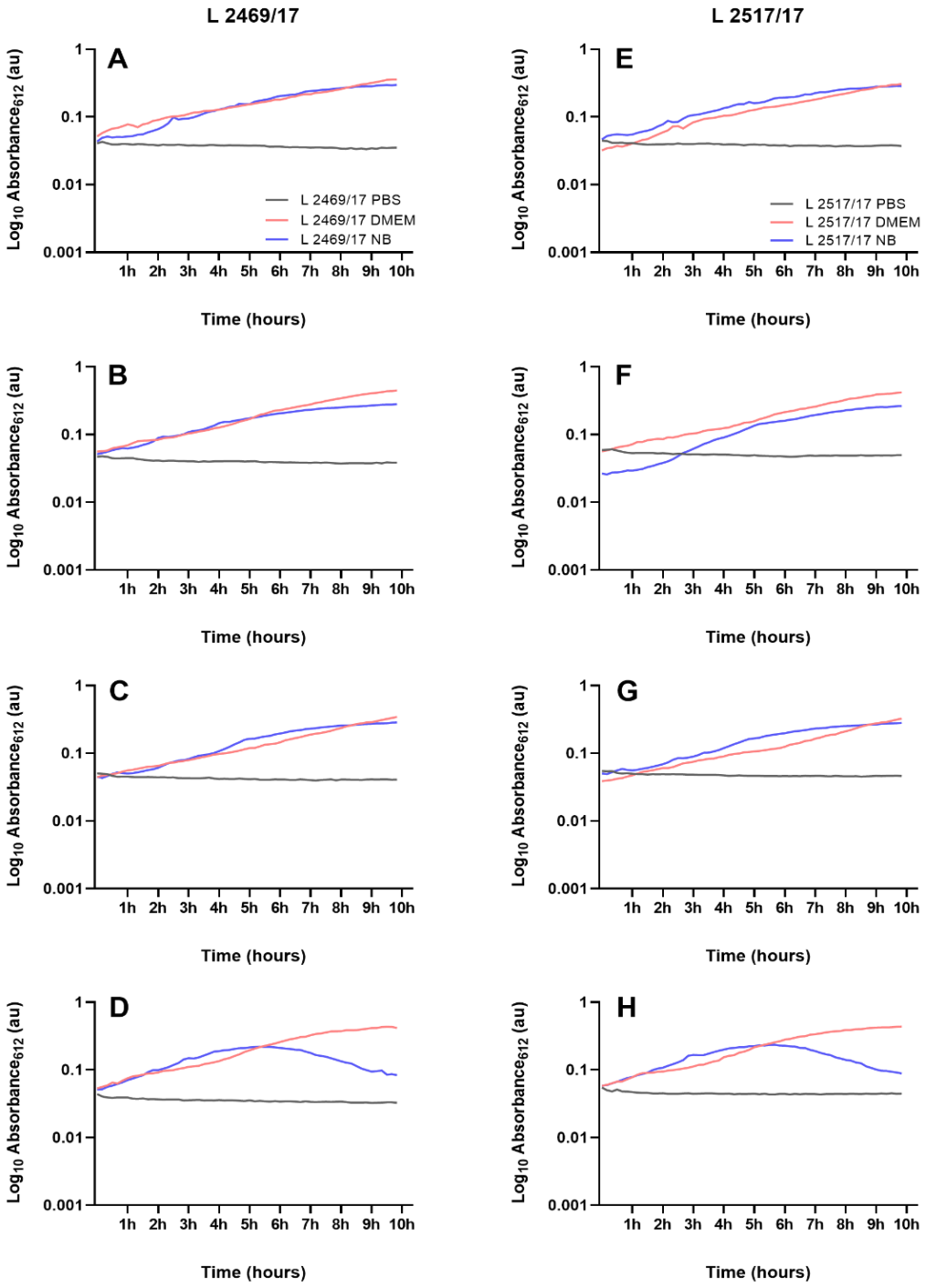


Figure 9.7 - Growth of S. Dublin isolates L 2469/17 (A-D) and L 2517/17 (E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

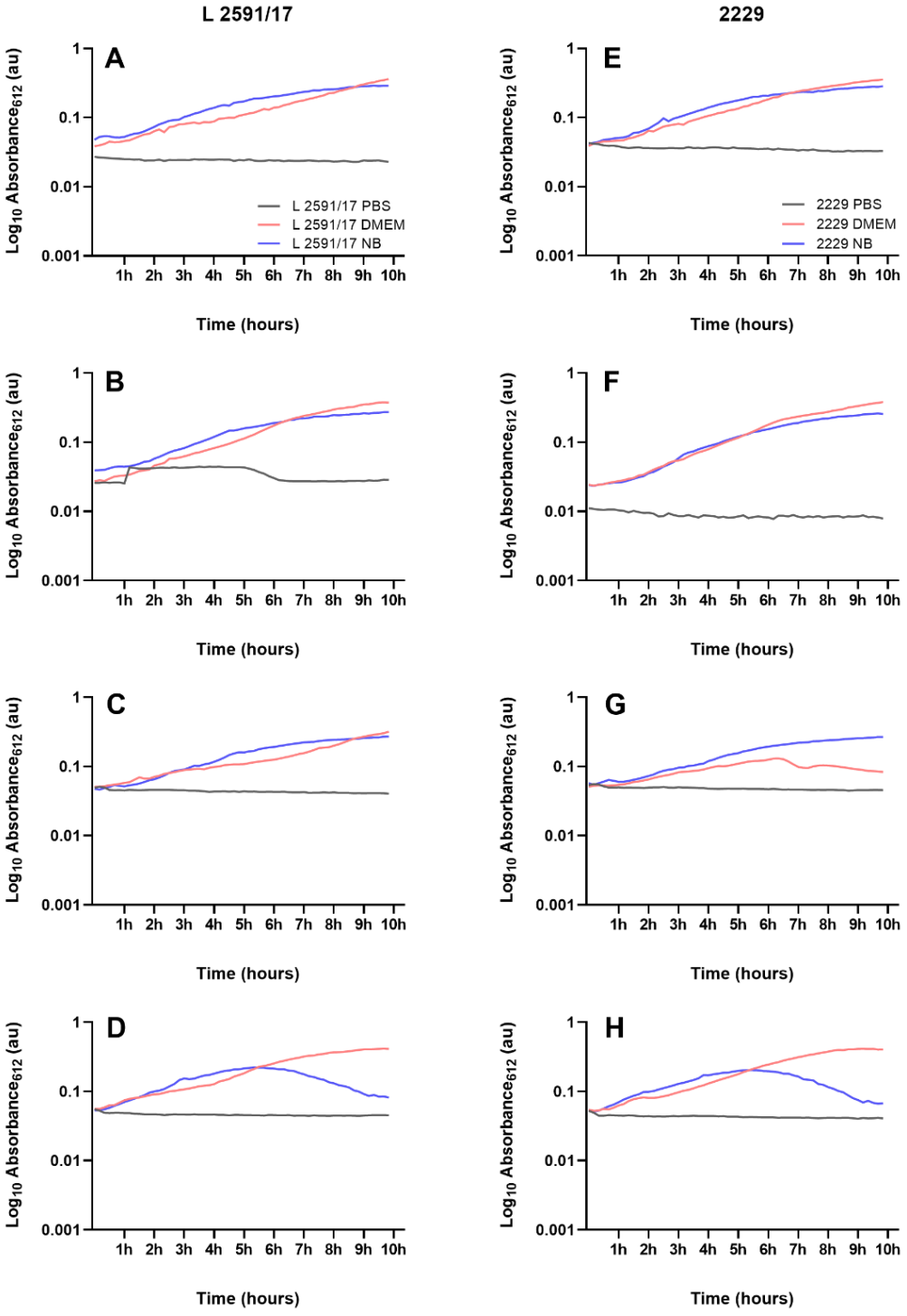


Figure 9.8 - Growth of S. Dublin isolates L 2591/17 (A-D) and 2229(E-H) in PBS, BCECM without antibiotics and NB. Isolates were grown in nutrient broth (NB – blue line) overnight in an orbital shaking incubator at 37°C to stationary phase. 100µl of these cultures were "washed" in phosphate buffered saline (PBS – grey line) and resuspended in 1ml of NB, PBS or bovine caruncular epithelial cell medium (BCECM – red line) without antibiotics. Absorbance at wavelength 612nm was measured every 10 minutes by the TECAN-96 well plate reader over the course of 10h.

Table 9.2 - Concentration of DNA extracted from *S.* Dublin isolates isolated from cattle for whole genome sequencing.

Isolate name	Concentration ng/μl	Final Volume μl
2229	6.8	50
L 1938/17	5.2	50
L 1941/17	10.2	50
L 2100/17	20.0	50
L 2104/17	8.6	50
L 2135/17	28.0	50
L 2160/17	18.6	50
L 2162/17	28.5	50
L 2185/17	23.5	50
L 2284/17	24.2	50
L 2294/17	15.7	50
L 2348/17	17.2	50
L 2424/17	11.5	50
L 2469/17	16.2	50
L 2517/17	11.6	50
L 2591/17	13.6	50

Isolates were grown overnight in nutrient broth in an orbital shaking incubator at 37°C to stationary phase. 1ml of these cultures was used in the process of DNA extraction using the QiAmp DNA Mini kit (QUIAGEN) and quantified using the Invitrogen Qubit dsDNA High Sensitivity Assay kit according to the manufacturers' instructions.

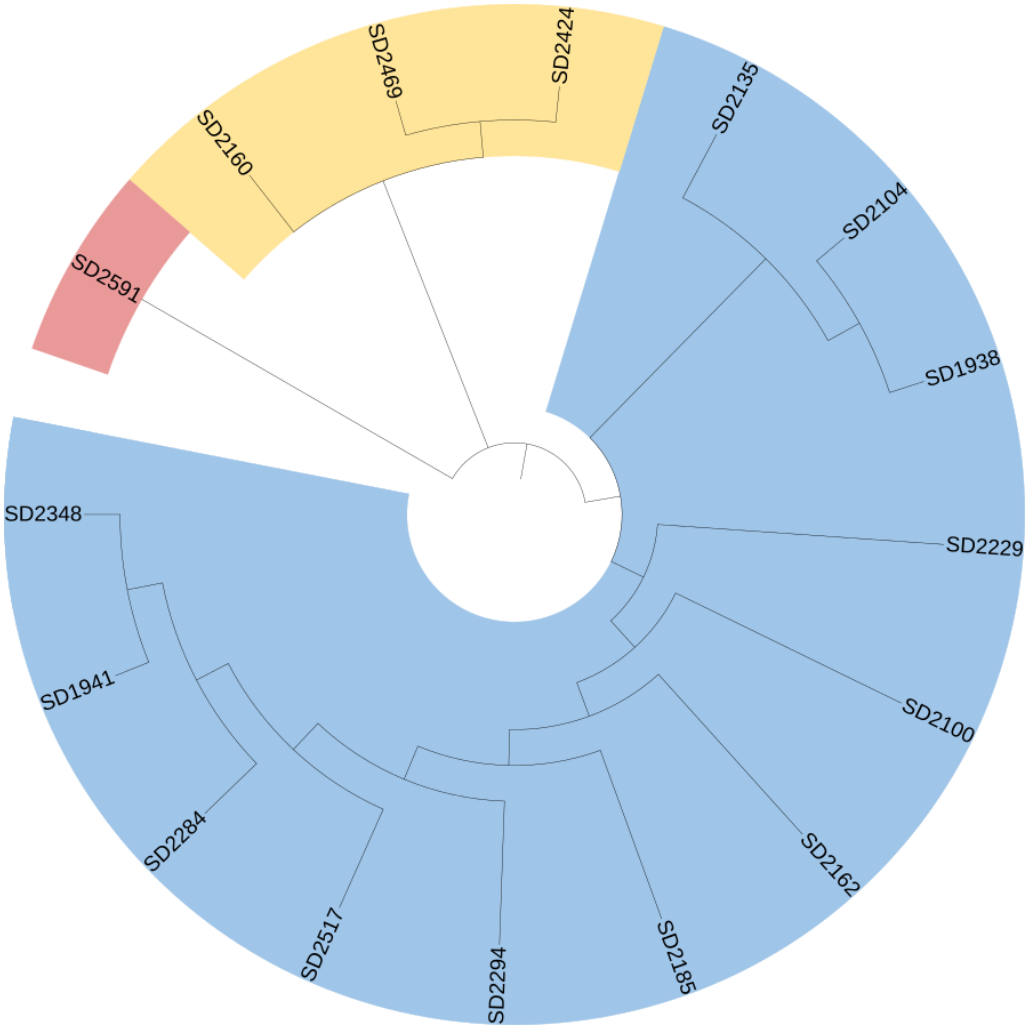


Figure 9.9 - Phylogenetic alignment of 16 *S.* Dublin isolates based on Whole Genome MLST. Whole genome Multi-Locus Sequence Type (MLST) was completed on a 95% loci presence, aligned using Roary by Dr Adam Blanchard, and mapped using iTol (Letunic and Bork, 2007; Page et al., 2015).Branch lengths have been inactivated on this tree to demonstrate where clades were originally rooted.

Table 9.3 - Virulence genes identified in 266 *S.* Dublin and 266 *S.* Typhimurium isolates. The whole genome sequences of all *S.* Dublin and *S.* Typhimurium isolates were compared to a database of known virulence genes in the Virulence Factor Database (VFDB) (Bo Liu et al., 2019). Genes were defined as being VFDB-inferred "present" if there was sequence homology of 90% or above. "16" denotes the 16 *S.* Dublin isolates used in phenotypic studies isolated from cattle, "266" refers to all 266 *S.* Dublin isolates used in the computational study including the original 16 isolates and "Ty" refers to the 266 *S.* Typhimurium isolates used in the computational study. "Y" denotes that the gene was present in at least one isolate in each subset, "N" means the gene was not identified in any isolates in the subset. "Cat" meaning "categorisation" 1 and 2.

16	266	Ty	Gene Name	Accession	Function	Cat 1	Cat 2	Reference
Y	Y	Y	*avrA*	NP_461786	Effector, Acetyltransferase (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000529
Y	Y	N	*hilA*	NP_461797	Invasion protein transcriptional activator	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000538
Y	Y	N	*hilC*	NP_461788	Invasion regulatory protein	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000531
Y	Y	N	*hilD*	NP_461796	Invasion protein regulatory protein	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000537
Y	Y	N	*iacP*	NP_461802	Putative acyl carrier protein	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254404
Y	Y	N	*iagB*	NP_461798	Invasion protein (putative)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254400
Y	Y	Y	*invA*	NP_461817	Major export apparatus protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000557
Y	Y	Y	*invB*	NP_461816	Chaperone (T3SS) SpaK	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254418
N	Y	Y	*invC*	NP_461815	ATPase	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000555
Y	Y	Y	*invE*	NP_461818	Gatekeeper (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000558
Y	Y	Y	*invF*	NP_461820	Regulatory protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000560
Y	Y	Y	*invG*	NP_461819	Secretin (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000559
Y	Y	Y	*invH*	NP_461821	Pilotin (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000561
Y	Y	Y	*invI*	NP_461814	Stalk protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000554

Y	Y	Y	*invJ*	NP_461813	Needle length regulator (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000553
Y	Y	Y	*orgA*	NP_461791	Accessory cytosolic protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG003645
Y	Y	Y	*orgB*	WP_000916654	Type 3 secretion system linker protein	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/protein/WP_000916654
Y	Y	Y	*orgC*	NP_461789	Effector protein (T3SS)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254391
Y	Y	Y	*prgH*	NP_461795	Outer MS ring protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000536
Y	Y	Y	*prgI*	NP_461794	Needle filament protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000535
Y	Y	Y	*prgJ*	NP_461793	Inner rod protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000534
Y	Y	Y	*prgK*	NP_461792	Inner MS ring (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000533
Y	Y	Y	*sicA*	NP_461807	Chaperone for SipC and SipB	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000547
Y	Y	Y	*sicP*	NP_461800	Chaperone for SptP	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000541
Y	Y	Y	*sipA*	NP_461803	Pathogenicity island 1 effector	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254405
Y	Y	Y	*sipB*	NP_461806	Hydrophilic translocator, pore protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000546
Y	Y	Y	*sipC*	NP_461805	Hydrophilic translocator, pore protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000545
Y	Y	Y	*sipD*	NP_461804	Hydrophilic translocator, needle tip protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000544
N	N	Y	*slrP*	NP_459778	leucine-rich repeat-containing protein, E3 ubiquitin ligase (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000469
Y	Y	Y	*sopA*	NP_461011	Similar to secreted effector protein of Salmonella Dublin, E3 ubiquitin protein ligase	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1253587
Y	Y	Y	*sopB/sigD*	NP_460064	Inositol phosphatase	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1252609
Y	Y	Y	*sopD2*	NP_459947	SPI1 protein	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1252490
Y	Y	Y	*sopD*	NP_461866	Secreted protein in the Sop family, transferred to eukaryotic cells	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254468

N	Y	Y	*sopE2*	NP_460811	T3SS protein	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1253374
Y	Y	Y	*spaO*	NP_461812	C ring protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000552
Y	Y	Y	*spaP*	NP_461811	Minor export apparatus protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000551
Y	Y	Y	*spaQ*	NP_461810	Minor export apparatus protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000550
Y	Y	Y	*spaR*	NP_461809	Minor export apparatus protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000549
Y	Y	Y	*spaS*	NP_461808	Export apparatus switch protein (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000548
Y	Y	Y	*sptP*	NP_461799	SPI1 effector protein	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254401
Y	Y	N	*spvA*	NP_490530	Outer membrane protein (T3SS)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1256200
Y	Y	Y	*spvB*	NP_490529	Effector (T3SS)	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000439
N	Y	Y	*spvC*	NP_490528	Hydrophilic protein (T3SS)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1256201
N	Y	Y	*spvR*	NP_490531	Regulation of spv operon (T3SS)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1256197
Y	Y	N	*rpoS*	NP_461845	RNA polymerase sigma factor (controls spv gene expression)	T3SS	SPI1	https://www.ncbi.nlm.nih.gov/gene/1254447
N	Y	N	*sprB*	NP_461787	Transcriptional regulator	T3SS	SPI1	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000530
N	N	Y	*gogB*	NP_461519	Type 3 secretion system effector, anti-inflammatory effector	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG004006
N	Y	Y	*pipB*	NP_460061	Effector protein	T3SS	SPI2	https://www.ncbi.nlm.nih.gov/gene/1252606
N	Y	Y	*pipB2*	WP_001738474	SPI2 effector protein	T3SS	SPI2	https://www.ncbi.nlm.nih.gov/protein/487626892/
Y	Y	Y	*sifA*	NP_460194	Effector protein	T3SS	SPI2	https://www.ncbi.nlm.nih.gov/gene/1252742
Y	Y	Y	*sifB*	NP_460561	Effector protein	T3SS	SPI2	https://www.ncbi.nlm.nih.gov/gene/1253120
Y	Y	N	*sopE*	WP_000182072	sopE2 - SPI1 T3SS guanine nucleotide exchange factor	T3SS	SPI2	https://www.ncbi.nlm.nih.gov/protein/446104217/
Y	Y	Y	*spiC/ssaB*	NP_460358	T3SS protein	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000494

N	Y	Y	*ssaC*	NP_460359	Secretin (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000495
Y	Y	Y	*ssaD*	NP_460360	Outer MS ring protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000496
Y	Y	Y	*ssaE*	NP_460361	Chaperone for sseB	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000497
N	N	Y	*ssaG*	NP_460371	Needle filament protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000507
Y	Y	Y	*ssaH*	NP_460372	T3SS protein	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000508
Y	Y	Y	*ssaI*	NP_460373	Inner rod protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000509
Y	Y	Y	*ssaJ*	NP_460374	Inner MS ring (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000510
Y	Y	Y	*ssaK*	NP_460376	Stator (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000511
Y	Y	Y	*ssaL*	NP_460377	Gatekeeper (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000512
Y	Y	Y	*ssaM*	NP_460378	Protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000513
Y	Y	Y	*ssaN*	NP_460380	ATPase (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000515
N	N	Y	*ssaO*	NP_460381	T3SS stalk protien	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000516
Y	Y	Y	*ssaP*	NP_460382	Needle length regulator (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000517
Y	Y	Y	*ssaQ*	NP_460383	C ring protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000518
Y	Y	Y	*ssaR*	NP_460384	Minor export apparatus protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000519
N	N	Y	*ssaS*	NP_460385	Minor export apparatus protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000520
Y	Y	Y	*ssaT*	NP_460386	Minor export apparatus protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000521
Y	Y	Y	*ssaU*	NP_460387	Export apparatus switch protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000522
Y	Y	Y	*ssaV*	NP_460379	Major export apparatus protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000514

Y	Y	Y	*sscA*	NP_460364	Chaperone for sseC (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000500
Y	Y	Y	*sscB*	NP_460368	Chaperone for sseF	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000504
Y	Y	Y	*sseA*	NP_460362	Chaperone for sseB and sseD (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000498
N	N	Y	*sseB*	NP_460363	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000499
Y	Y	Y	*sseC*	NP_460365	Hydrophilic translocator, pore protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000501
Y	Y	Y	*sseD*	NP_460366	Hydrophilic translocator, pore protein (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000502
Y	Y	Y	*sseE*	NP_460367	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000503
Y	Y	Y	*sseF*	NP_460369	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000505
N	Y	Y	*sseG*	NP_460370	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000506
Y	Y	Y	*sseI/srfH*	NP_460026	Effector, cysteine protease (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG003971
Y	Y	Y	*sseJ*	NP_460590	Effector, glycerophospholipid, cholesterolacyltransferase (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000471
Y	Y	Y	*sseK1*	NP_463026	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG004016
Y	Y	Y	*sseK2*	NP_461081	Effector (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG003982
Y	Y	Y	*sseL*	NP_461229	Effector, deubiquitinase (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG003977
N	N	Y	*sspH1*	AAD40326	Effector (T3SS), E3 upiquitin ligase	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG042216
Y	Y	Y	*sspH2*	NP_461184	Effector, E3 ubiquitin ligase	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000468
Y	Y	N	*ssrA*	NP_460357	Sensor kinase (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000493
Y	Y	N	*ssrB*	NP_460356	Transcriptional activator (T3SS)	T3SS	SPI2	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000492
N	N	Y	*misL*	NP_462656	Putative autotransporter	T5SS	SPI3	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG002304

Y	Y	N	*icmH/dotU*	WP_000343978	Type IVB secretion system protein icmH/dotU	T6SS	icmH/dot	https://www.ncbi.nlm.nih.gov/gene/66755852
Y	Y	N	STMDT2_RS01405	WP_000312802	T6SS tube protein	T6SS	hcp	https://www.ncbi.nlm.nih.gov/protein/446234947/
Y	Y	N	SG_RS05215	WP_001284964	Hcp family T6SS effector	T6SS	hcp	https://www.ncbi.nlm.nih.gov/protein/WP_001284964
N	Y	N	SG_RS05300	WP_001142966	Hcp family T6SS effector	T6SS	hcp	https://www.ncbi.nlm.nih.gov/protein/447065710/
Y	Y	N	*impA*	WP_000367626	impA family type 6 secretion system protein	T6SS	imp	https://www.ncbi.nlm.nih.gov/protein/446289771/
Y	Y	N	*sciQ*	WP_000976553	Membrane protein	T6SS	sci	https://www.ncbi.nlm.nih.gov/protein/446899297/
Y	Y	N	SG1030	WP_000076069	T6SS impA family N-terminal domain-containing protein	T6SS	imp	https://www.ncbi.nlm.nih.gov/protein/445998214/
Y	Y	N	*tagH*	WP_000796942	T6SS associated FHA domain protein	T6SS	tag	https://www.ncbi.nlm.nih.gov/protein/446719629/
Y	Y	N	*tagK*	WP_000806681	T6SS associated protein	T6SS	tag	https://www.ncbi.nlm.nih.gov/protein/446729368/
Y	Y	N	*tagO*	WP_000089148	T6SS associated protein	T6SS	tag	https://www.ncbi.nlm.nih.gov/protein/446011293/
Y	Y	N	*tssA*	WP_001752219	T6SS protein	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/487662354/
Y	Y	N	*tssB*	WP_000996817	T6SS contractile sheath small subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446919561/
N	Y	N	*tssB*	WP_000031252	T6SS contractile sheath small subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/445953397/
Y	Y	N	*tssC*	WP_000013880	T6SS contractile sheath large subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/445936025/
Y	Y	N	*tssC*	WP_000058001	T6SS contractile sheath large subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/445980146/
Y	Y	N	*tssE*	WP_000108007	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446030152/
Y	Y	N	*tssF*	WP_000371510	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446293655/
Y	Y	N	*tssF*	WP_000393869	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446316014/
Y	Y	N	*tssG*	WP_000145244	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446067389/
Y	Y	N	*tssG*	WP_000509049	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446431194/
Y	Y	N	*tssH*	WP_000449797	T6SS ATPase	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446371942/
Y	Y	N	*tssJ*	WP_001007106	T6SS lipoprotein	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446929850/

Y	Y	N	*tssJ*	WP_124084121	T6SS lipoprotein	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/1524033542/
Y	Y	N	*tssK*	WP_000118732	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446040877/
Y	Y	N	*tssK*	WP_000246454	T6SS baseplate subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446168599/
Y	Y	N	*tssL*	WP_000132483	T6SS protein	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/446054628/
Y	Y	N	*tssM*	WP_001168956	T6SS membrane subunit	T6SS	tss	https://www.ncbi.nlm.nih.gov/protein/447091700/
N	Y	N	*vgrG*	WP_000103449	T6SS tip protein	T6SS	vgr	https://www.ncbi.nlm.nih.gov/protein/446025594/
Y	Y	N	*vgrS*	WP_000095505	T6SS tip protein	T6SS	vgr	https://www.ncbi.nlm.nih.gov/protein/WP_000095505.1/
Y	Y	N	*pagN*	WP_000787603	Adhesin/invasin protein	Adherence	Outer membrane protein	https://www.ncbi.nlm.nih.gov/protein/WP_000787603
Y	Y	Y	*shdA*	NP_461448	AIDA autotransporter-like protein	Adherence	Outer membrane protein	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG002305
Y	Y	N	*siiE*	WP_000527219	Non-fimbrial adhesin	Adherence	Outer-membrane	https://www.ncbi.nlm.nih.gov/protein/446449364/
Y	Y	Y	*sinH*	NP_461452	Intimin-like protein	Adherence	Colonisation factor	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG002307
Y	Y	Y	*mig-14*	NP_461708	Antimicrobial resistance protein	Antimicrobial Resistance	AMR	https://pubmed.ncbi.nlm.nih.gov/15661016/
Y	Y	N	*cheA*	NP_460878	Sensory histidine protein kinase	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253442
Y	Y	N	*cheB*	NP_460874	Chemotaxis response regulator protein - glutamate methyltransferase	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253438
Y	Y	N	*cheR*	NP_460875	Chemotaxis protein - glutamate O-methyltransferase	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253439
Y	Y	Y	*cheW*	NP_460877	Chemotaxis protein	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253441
Y	Y	Y	*cheY*	NP_460873	Two-component system response regulator	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253437
Y	Y	N	*cheZ*	NP_460872	Protein phosphatase	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253436
Y	Y	N	*tar/cheM*	NP_460876	Methyl-accepting chemotaxis protein II	Chemotaxis	che	https://www.ncbi.nlm.nih.gov/gene/1253440

Y	Y	Y	*csgA*	NP_460115	Curlin major subunit	Curli	csg	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000458
Y	Y	Y	*csgB*	NP_460114	Minor curlin subunit precursor, curli nucleator protein	Curli	csg	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000457
N	N	Y	*csgC*	NP_460116	Curli assembly protein	Curli	csg	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000459
N	Y	Y	*csgD*	NP_460113	Transcriptional regulator	Curli	csg	https://www.ncbi.nlm.nih.gov/gene/1252660
Y	Y	Y	*csgE*	NP_460112	Curli production assembly/transport protein	Curli	csg	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000460
N	Y	Y	*csgF*	NP_460111	Curli production assembly/transport protein	Curli	csg	mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000461
Y	Y	Y	*csgG*	NP_460110	Curli production assembly/transport protein	Curli	csg	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000462
Y	Y	N	DcrB	WP_000375832	DcrB-related protein	dcrB	dcrB	https://www.ncbi.nlm.nih.gov/protein/446297977/
Y	Y	N	DUF2094	WP_001254137	DUF2094 domain-containing protein	DUF	DUF	https://www.ncbi.nlm.nih.gov/protein/447176881/
Y	Y	N	DUF2195	WP_001596567	DUF2195 family protein	DUF	DUF	https://www.ncbi.nlm.nih.gov/protein/486367205/
Y	Y	N	DUF2778	WP_000968384	DUF2778 domain-containing protein	DUF	DUF	https://www.ncbi.nlm.nih.gov/protein/446891128/
Y	Y	N	*fur*	NP_459678	Transcriptional repressor of iron-responsive genes (ferric regulator)	Ferric uptake	fur	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000478
N	N	Y	*entA*	NP_459590	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	Ferric uptake	ent	https://www.ncbi.nlm.nih.gov/gene/1252118
N	N	Y	*entB*	NP_459589	Isochorismatase - a catalyst in Siderophore biosynthesis	Ferric uptake	ent	https://www.ncbi.nlm.nih.gov/gene/1252117
N	N	Y	*entC*	NP_459587	Isochorismate synthase	Ferric uptake	ent	https://www.ncbi.nlm.nih.gov/gene/1252115
N	N	Y	*entE*	NP_459588	2,3-dihydroxybenzoate-AMP ligase	Ferric uptake	ent	https://www.ncbi.nlm.nih.gov/gene/1252116
N	N	Y	*entS*	WP_001081661	Enterobactin exporter	Ferric uptake	ent	https://www.ncbi.nlm.nih.gov/gene/11839529
N	N	Y	*fepA*	NP_459577	Outer membrane receptor protein	Ferric uptake	fep	https://www.ncbi.nlm.nih.gov/gene/1252105

N	N	Y	*fepB*	NP_459586	Fe2+-enterobactin ABC transporter substrate-binding protein	Ferric uptake	fep	https://www.ncbi.nlm.nih.gov/gene/1252114
N	N	Y	*fepC*	WP_000140620	Ferric enterobactin transport ATP-binding protein	Ferric uptake	fep	https://www.ncbi.nlm.nih.gov/gene/17157740
N	N	Y	*fepD*	WP_001277880	Ferric enterobactin transport protein	Ferric uptake	fep	https://www.ncbi.nlm.nih.gov/gene/17157741
N	N	Y	*fepG*	WP_000480067	Ferric enterobactin transport protein	Ferric uptake	fep	https://www.ncbi.nlm.nih.gov/gene/17157738
Y	Y	Y	*iroB*	NP_461700	Putative glycosyltransferase	Ferric uptake	iro	https://www.ncbi.nlm.nih.gov/gene/1254296
Y	Y	Y	*iroC*	NP_461701	Multidrug ABC transporter ATP-binding protein	Ferric uptake	iro	https://www.ncbi.nlm.nih.gov/gene/1254297
Y	Y	N	*iroD*	NP_461702	Enterochelin esterase	Ferric uptake	iro	https://www.ncbi.nlm.nih.gov/gene/1254298
Y	Y	N	*iroE*	NP_461703	Hydrolase	Ferric uptake	iro	https://www.ncbi.nlm.nih.gov/gene/1254299
Y	Y	Y	*iroN*	NP_461704	TonB-dependent siderophore receptor protein	Ferric uptake	iro	https://www.ncbi.nlm.nih.gov/gene/1254300
N	N	Y	*iucA*	WP_000602863	Aerobactin siderophore biosynthesis protein (siderophore synthetase component)	Ferric uptake	iuc	https://www.ncbi.nlm.nih.gov/gene/11842790
N	N	Y	*iucB*	WP_000011908	Aerobactin siderophore biosynthesis protein (siderophore synthetase component)	Ferric uptake	iuc	https://www.ncbi.nlm.nih.gov/gene/11842789
N	N	Y	*iucC*	WP_001015721	Siderophore synthetase component	Ferric uptake	iuc	https://www.ncbi.nlm.nih.gov/gene/11842788
N	N	Y	*iucD*	WP_000750130	L-lysine 6-monooxygenase protein	Ferric uptake	iuc	https://www.ncbi.nlm.nih.gov/gene/13923533
N	N	Y	*iutA*	YP_006955515	Aerobactin siderophore ferric receptor	Ferric uptake	iut	https://www.ncbi.nlm.nih.gov/gene/13912881
Y	Y	N	*bcfA*	NP_459026	Fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251539
N	Y	N	*bcfB*	NP_459027	Fimbrial chaperone protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251540
Y	Y	N	*bcfC*	NP_459028	Fimbrial outer membrane usher protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251541

Y	Y	N	*bcfD*	NP_459029	Fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251542
Y	Y	N	*bcfE*	NP_459030	Fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251543
Y	Y	N	*bcfF*	NP_459031	Fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251544
Y	Y	N	*bcfG*	NP_459032	Fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251545
Y	Y	N	*bcfH*	NP_459033	Hypothetical fimbrial protein	Fimbriae	bcf	https://www.ncbi.nlm.nih.gov/gene/1251546
Y	Y	N	*fimA*	NP_459538	Type I fimbriae major pillin	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000443
Y	Y	Y	*fimC*	NP_459540	Chaperone protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000445
Y	Y	Y	*fimD*	NP_459541	Usher protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000446
Y	Y	Y	*fimF*	NP_459543	Type I fimbriae adaptor protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000448
Y	Y	Y	*fimH*	NP_459542	Type I fimbriae minor fimbrial subunit, adhesin	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000447
Y	Y	Y	*fimI*	NP_459539	Fimbrial protein internal segment	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000444
Y	Y	N	*fimW*	NP_459547	Putative fimbrial protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000451
Y	Y	N	*fimY*	NP_459545	Putative regulatory protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000450
Y	Y	N	*fimZ*	NP_459544	Fimbrial protein	Fimbriae	fim	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000449
Y	Y	Y	*lpfA*	NP-462541	Long polar fimbria protein	Fimbriae	lpf	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000456
Y	Y	Y	*lpfB*	NP_462540	Long polar fimbrial chaperone protein	Fimbriae	lpf	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000455
Y	Y	Y	*lpfC*	NP_462539	Long polar fimbrial usher protein	Fimbriae	lpf	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000454
Y	Y	Y	*lpfD*	NP_462538	Long polar fimbrial protein	Fimbriae	lpf	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000453
Y	Y	Y	*lpfE*	NP_462537	Long polar fimbrial minor subunit, adhesin	Fimbriae	lpf	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000452
N	N	Y	*pefA*	NP_490510	Plasmid-encoded fimbriae major subunit	Fimbriae	pef	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000435

Y	Y	Y	*pefB*	NP_490511	Plasmid-encided fimbriae regulatory protein	Fimbriae	pef	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000436
N	N	Y	*pefC*	NP_490509	Plasmid-encoded fimbriae usher protein	Fimbriae	pef	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000434
N	N	Y	*pefD*	NP_490508	Plasmid-encoded fimbriae chaperone protein	Fimbriae	pef	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000433
Y	Y	N	*pegB*	WP_000871808	Fimbrial assembly chaperone	Fimbriae	peg	https://www.ncbi.nlm.nih.gov/protein/446794552/
N	Y	N	*pegC*	WP_000413358	Fimbrial biogenesis outer membrane usher protein	Fimbriae	peg	https://www.ncbi.nlm.nih.gov/protein/446335503/
Y	Y	N	*pegD*	WP_000825916	Fimbrial protein	Fimbriae	peg	https://www.ncbi.nlm.nih.gov/protein/446748660/
Y	Y	N	*safA*	NP_459297	Putative outer membrane protein	Fimbriae	saf	https://www.ncbi.nlm.nih.gov/gene/1251818
Y	Y	N	*safB*	NP_459298	Putative fimbriae assembly chaperone	Fimbriae	saf	https://www.ncbi.nlm.nih.gov/gene/1251819
Y	Y	N	*safC*	NP_459299	Pilin outer membrane usher protein	Fimbriae	saf	https://www.ncbi.nlm.nih.gov/gene/1251820
N	Y	N	*safD*	NP_459300	Structural protein	Fimbriae	saf	https://www.ncbi.nlm.nih.gov/gene/1251821
Y	Y	N	*sefA*	WP_001674837	SEF14 fimbria major subunit	Fimbriae	sef	https://www.ncbi.nlm.nih.gov/protein/487406756/
Y	Y	N	*sefC*	WP_000753916	SEF14/18 fimbria usher protein	Fimbriae	sef	https://www.ncbi.nlm.nih.gov/protein/446676570/
Y	Y	N	*sefD*	WP_001077813	SEF18 fimbria major subunit	Fimbriae	sef	https://www.ncbi.nlm.nih.gov/protein/447000557/
Y	Y	N	*sefR*	WP_164920268	helix-turn-helix domain-containing protein	Fimbriae	sef	https://www.ncbi.nlm.nih.gov/protein/1818933223/
Y	Y	N	*stbA*	WP_012443535	Hypothetical protein	Fimbriae	stb	https://www.ncbi.nlm.nih.gov/gene/1251859
Y	Y	N	*stbB*	WP_012443534	Hypothetical protein	Fimbriae	stb	https://www.ncbi.nlm.nih.gov/gene/1251858
Y	Y	N	*stbC*	WP_181409690	Hypothetical protein	Fimbriae	stb	https://www.ncbi.nlm.nih.gov/gene/1251857
Y	Y	N	*stbD*	NP_459332	Fimbrial protein	Fimbriae	stb	https://www.ncbi.nlm.nih.gov/gene/1251856
Y	Y	N	*stbE*	NP_459331	Fimbrial assembly protein	Fimbriae	stb	https://www.ncbi.nlm.nih.gov/gene/1251855
Y	Y	N	*stdA*	NP_461946	Putative fimbrial-like protein	Fimbriae	std	https://www.ncbi.nlm.nih.gov/gene/1254552
Y	Y	N	*stdB*	NP_461945	Fimbrial protein	Fimbriae	std	https://www.ncbi.nlm.nih.gov/gene/1254551
Y	Y	N	*stdC*	NP_461944	Fimbrial chaperone protein	Fimbriae	std	https://www.ncbi.nlm.nih.gov/gene/1254550
Y	Y	Y	*steA*	NP_460542	Fimbrial protein	Fimbriae	ste	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG042067

Y	Y	Y	*steB*	AAL20547	Fimbrial protein	Fimbriae	ste	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG042214
Y	Y	Y	*steC*	NP_460656	Fimbrial protein	Fimbriae	ste	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG042066
N	Y	N	*steD*	WP_000178263	Fimbrial protein	Fimbriae	ste	https://www.ncbi.nlm.nih.gov/protein/446100408/
Y	Y	N	*steE*	WP_000832400	Fimbrial protein	Fimbriae	ste	https://www.ncbi.nlm.nih.gov/protein/446755144/
Y	Y	N	*steF*	WP_001079653	Fimbrial protein	Fimbriae	ste	https://www.ncbi.nlm.nih.gov/protein/447002397/
Y	Y	N	*stfC*	NP_459201	Outer membrane usher protein	Fimbriae	stf	https://www.ncbi.nlm.nih.gov/gene/1251714
N	Y	N	*stfD*	NP_459202	Fimbrial protein	Fimbriae	stf	https://www.ncbi.nlm.nih.gov/gene/1251715
Y	Y	N	*stfE*	NP_459203	Fimbrial protein	Fimbriae	stf	https://www.ncbi.nlm.nih.gov/gene/1251716
N	Y	N	*stfF*	NP_459204	Fimbrial protein	Fimbriae	stf	https://www.ncbi.nlm.nih.gov/gene/1251717
Y	Y	N	*stfG*	NP_459205	Fimbrial protein	Fimbriae	stf	https://www.ncbi.nlm.nih.gov/gene/1251718
Y	Y	N	*sthA*	NP_463450	Fimbrial assembly chaperone	Fimbriae	sth	https://www.ncbi.nlm.nih.gov/gene/1256120
N	Y	N	*sthB*	NP_463449	Putative fimbrial usher protein	Fimbriae	sth	https://www.ncbi.nlm.nih.gov/gene/1256119
Y	Y	N	*sthC*	WP_001160713	Fimbrial outer membrane usher protein	Fimbriae	sth	https://www.ncbi.nlm.nih.gov/protein/447083457/
Y	Y	N	*sthD*	NP_463448	Fimbrial protein	Fimbriae	sth	https://www.ncbi.nlm.nih.gov/gene/1256118
Y	Y	N	*sthE*	NP_463447	Major fimbrial protein	Fimbriae	sth	https://www.ncbi.nlm.nih.gov/gene/1256117
Y	Y	N	*stiA*	NP_459182	Fimbrial protein	Fimbriae	sti	https://www.ncbi.nlm.nih.gov/gene/1251695
Y	Y	N	*stiB*	NP_459181	Long polar fimbrial chaperone lpfB	Fimbriae	sti	https://www.ncbi.nlm.nih.gov/gene/1251694
Y	Y	N	*stiC*	NP_459180	Fimbrial assembly protein	Fimbriae	sti	https://www.ncbi.nlm.nih.gov/gene/1251693
Y	Y	N	*stiH*	NP_459179	Fimbrial protein	Fimbriae	sti	https://www.ncbi.nlm.nih.gov/gene/1251692
Y	Y	N	*yehD*	WP_000830690	Fimbrial protein	Fimbriae	yeh	https://www.ncbi.nlm.nih.gov/protein/WP_000830690
N	N	Y	*gtrB*	WP_000703614	Bactoprenol glucosyl transferase	Flippase	gtr	https://www.ncbi.nlm.nih.gov/gene/17155475
Y	Y	N	*flgA*	NP_460144	Flagellar basal body P-ring formation protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252691
Y	Y	N	*flgB*	NP_460145	Flagellar basal body rod protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252692

Y	Y	N	*flgC*	NP_460146	Flagellar basal body rod protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252693
Y	Y	N	*flgD*	NP_460147	Flagellar basal body rod modification protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252694
Y	Y	N	*flgE*	NP_460148	Flagellar hook protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252695
Y	Y	N	*flgF*	NP_460149	Flagellar biosynthesis protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252696
Y	Y	Y	*flgG*	NP_460150	Flagellar basal body rod protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252697
Y	Y	N	*flgH*	NP_460151	Flagellar basal body L-ring protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252698
Y	Y	N	*flgI*	NP_460152	Flagellar biosynthesis protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252699
Y	Y	N	*flgJ*	NP_460153	Flagellar rod assembly protein, muramidase	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252700
Y	Y	N	*flgK*	NP_460154	Flagellar hook-associated protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252701
Y	Y	N	*flgL*	NP_460155	Flagellar hook-filament junction protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252702
Y	Y	N	*flgM*	NP_460143	Anti-sigma factor	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252690
Y	Y	N	*flgN*	NP_460142	Flagellar biosynthesis protein	Flagella	flg	https://www.ncbi.nlm.nih.gov/gene/1252689
Y	Y	N	*flhA*	NP_460870	Flagellar biosynthesis protein	Flagella	flh	https://www.ncbi.nlm.nih.gov/gene/1253434
Y	Y	N	*flhB*	NP_460871	Flagellar biosynthesis protein	Flagella	flh	https://www.ncbi.nlm.nih.gov/gene/1253435
Y	Y	Y	*flhC*	NP_460881	Regulator of flagellar biosynthesis	Flagella	flh	https://www.ncbi.nlm.nih.gov/gene/1253445
Y	Y	N	*flhD*	NP_460882	Regulator of flagellar biosynthesis	Flagella	flh	https://www.ncbi.nlm.nih.gov/gene/1253446
Y	Y	N	*flhE*	NP_460869	Flagellar protein	Flagella	flh	https://www.ncbi.nlm.nih.gov/gene/1253433
Y	Y	Y	*fliA*	NP_460909	RNA polymerase sigma factor	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253477
Y	Y	N	*fliB*	NP_460911	Lysine-N-methylase	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253479
Y	Y	N	*fliE*	NP_460921	Flagellar hook-basal body complex protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253489
Y	Y	N	*fliF*	NP_460922	Flagellar M-ring protien	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253490
Y	Y	Y	*fliG*	NP_460923	Flagellar motor switch protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253491
Y	Y	N	*fliH*	NP_460924	Flagellar assembly protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253492

Y	Y	N	*fliI*	NP_460925	Flagellum-specific ATP synthase	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253493
Y	Y	N	*fliJ*	NP_460926	Flagellar biosynthesis chaperone	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253494
Y	Y	N	*fliK*	NP_460927	Flagellar hook-length control protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253495
Y	Y	N	*fliL*	NP_460928	Flagellar basal body-associated protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253496
Y	Y	Y	*fliM*	NP_460929	Flagellar motor switch protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253497
Y	Y	N	*fliN*	NP_460930	Flagellar motor switch protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253498
Y	Y	N	*fliO*	NP_460931	Flagellar biosynthesis protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253499
Y	Y	Y	*fliP*	NP_460932	Flagellar biosynthetic protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253500
Y	Y	N	*fliQ*	NP_460933	Flagellar export apparatus protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253501
Y	Y	N	*fliR*	NP_460934	Flagellar biosynthetic protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253502
Y	Y	N	*fliS*	NP_460914	Flagellar export chaperone	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253482
Y	Y	N	*fliT*	NP_460915	Flagellar biosynthesis protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253483
Y	Y	N	*fliY*	NP_460907	Cystine ABC transporter substrate-binding protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253475
Y	Y	N	*fliZ*	NP_460908	Flagellar regulatory protein	Flagella	fli	https://www.ncbi.nlm.nih.gov/gene/1253476
Y	Y	N	*flk*	NP_461313	Flagella biosynthesis regulator	Flagella	flk	https://www.ncbi.nlm.nih.gov/gene/1253893
Y	Y	N	*motA*	NP_460880	Flagellar motor stator protein	Flagella	mot	https://www.ncbi.nlm.nih.gov/gene/1253444
Y	Y	N	*motB*	NP_460879	Flagellar motor protein	Flagella	mot	https://www.ncbi.nlm.nih.gov/gene/1253443
Y	Y	Y	*mgtB*	NP_462662	Magnesium-translocating P-type ATPase	Magnesium	SPI3	https://www.ncbi.nlm.nih.gov/gene/1255287
Y	Y	Y	*mgtC*	NP_462663	Magnesium transporter	Magnesium	SPI3	https://www.ncbi.nlm.nih.gov/gene/1255288
Y	Y	Y	*grvA*	YP_009223633	Similar to phage Gifsy-2	Phage	Phage	https://www.ncbi.nlm.nih.gov/gene/26794771
N	N	Y	*sodCI*	WP_000877926	Phage encoded superoxide dismutase precurser	Phage	Phage	https://www.ncbi.nlm.nih.gov/gene/17155636
N	N	Y	*rck*	NP_490501	Resistance to complement killing	Plasmid	Plasmid	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000442

N	Y	N	*pilM*	YP_009023794	Conjugal transfer protein	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983859
N	Y	N	*pilN*	YP_009023793	Pilus secretin (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983860
N	Y	N	*pilO*	YP_009023792	Pilus biogenesis protein (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983861
N	Y	N	*pilP*	YP_009023791	Pilus biogenesis protein (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983862
N	Y	N	*pilQ*	YP_009023790	ATP-binding protein (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983863
N	Y	N	*pilR*	YP_009023789	Integral membrane protein (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983864
N	Y	N	*pilS*	YP_009023788	Prepilin (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983865
N	Y	N	*pilT*	YP_009023787	Putative transglycosylate (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983866
N	Y	N	*pilU*	YP_009023786	Secretion leader peptidase/N-methyltransferase (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983867
N	Y	N	*pilV*	YP_009023785	Prepolin (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/18983872
N	Y	N	*pilV2*	NP_458639	Prepilin alternative C-terminal region (T4SS)	Plasmid pSTM709	Pilus	https://www.ncbi.nlm.nih.gov/gene/1250758
Y	Y	N	*mig-5*	YP_006956819	Putative carbonic anhydrase	Plasmid	pSENV	https://www.ncbi.nlm.nih.gov/gene/13911783
N	Y	N	*phoQ*	NP_460200	Sensor protein	Sensor	Sensor	https://www.ncbi.nlm.nih.gov/gene/1252748
Y	Y	N	*sciR*	WP_000227044	Shiga toxin A subunit	Toxin	sci	https://www.ncbi.nlm.nih.gov/protein/446149189/
Y	Y	N	SG1045	YP_002226104	RHS repeat protein	Toxin	RHS repeat	https://www.ncbi.nlm.nih.gov/gene/6922203
Y	Y	N	SG1048	WP_000509054	RHS repeat protein	Toxin	RHS repeat	https://www.ncbi.nlm.nih.gov/protein/446431199/
N	N	Y	*ompA*	NP_460044	Porin - outer membrane protein a precursor	Structural	Outer membrane protein	https://www.ncbi.nlm.nih.gov/gene/1252588
Y	Y	N	*ompD*	WP_000769035	Porin	Structural	Outer membrane protein	https://www.ncbi.nlm.nih.gov/protein/WP_000769035
Y	Y	N	*apeE*	NP_459562	Autotransporter outer membrane beta-barrel domain-containing protein	Structural	Outer membrane protein	https://www.ncbi.nlm.nih.gov/gene/?term=STM0570

N	Y	N	*tviB*	WP_000466893	Vi polysaccharide biosynthesis UDP-N-acetylglucosamine C-6 dehydrogenase	Vi	tvi	https://www.ncbi.nlm.nih.gov/protein/446389038/
N	Y	N	*tviC*	WP_000127915	Vi polysaccharide biosynthesis UDP-N-acetylglucosaminuronic acid C-4 epimerase	Vi	tvi	https://www.ncbi.nlm.nih.gov/protein/446050060/
Y	Y	N	*tviD*	WP_010989299	Vi polysaccharide biosynthesis protein	Vi	tvi	https://www.ncbi.nlm.nih.gov/protein/499298349/
Y	Y	N	*tviE*	WP_000632615	Vi polysaccharide biosynthesis glycosyltransferase	Vi	tvi	https://www.ncbi.nlm.nih.gov/protein/446555269/
N	Y	N	*vexA*	WP_000720235	Vi polysaccharide ABC transporter protein	Vi	vex	https://www.ncbi.nlm.nih.gov/protein/446642889/
Y	Y	N	*vexB*	WP_001023498	Vi polysaccharide ABC transporter inner membrane protein	Vi	vex	https://www.ncbi.nlm.nih.gov/protein/446946242/
N	Y	N	*vexC*	WP_000467404	Vi polysaccharide ABC transporter ATP-binding protein	Vi	vex	https://www.ncbi.nlm.nih.gov/protein/446389549/
N	Y	N	*vexD*	WP_000431675	Vi polysaccharide ABC transporter inner membrane protein	Vi	vex	https://www.ncbi.nlm.nih.gov/protein/WP_000431675
N	Y	N	*vexE*	NP_458730	Vi polysaccharide export protein	Vi	vex	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG000423
N	N	Y	*nleC*	WP_000704096	Hypothetical Protein	Hypothetical Protein	Hypothetical Protein	https://www.ncbi.nlm.nih.gov/gene/11836795
N	Y	N	*pilK*	WP_001330804	Hypothetical protein	Hypothetical protein	pil	https://www.ncbi.nlm.nih.gov/protein/WP_001330804.1?
Y	Y	N	*sciE*	WP_000750535	Hypothetical protein	Hypothetical protein	Hypothetical protein	https://www.ncbi.nlm.nih.gov/protein/446673189/
N	Y	N	*sciJ*	WP_014344502	Hypothetical protein	Hypothetical protein	Hypothetical protein	https://www.ncbi.nlm.nih.gov/protein/504110516/
Y	Y	N	SG1047	WP_000622532	Hypothetical protein	Hypothetical protein	Hypothetical protein	https://www.ncbi.nlm.nih.gov/protein/446545186/
Y	Y	N	SG1049	WP_001574177	Hypothetical protein	Hypothetical protein	Hypothetical protein	https://www.ncbi.nlm.nih.gov/protein/486295547/
Y	Y	Y	*ratB*	NP_461449	Putative outer membrane protein	Hypothetical protein	Hypothetical protein	http://www.mgc.ac.cn/cgi-bin/VFs/gene.cgi?GeneID=VFG002306

Y	Y	N	*stdD*	No record available

Table 9.4 - Metadata of 250 *S.* Dublin isolates downloaded from Enterobase and used in comparative analysis.

Uberstrain	Source	Collection Year
SAL_BA7444AA	Human	14
SAL_BA7951AA	Human	14
SAL_BA9360AA	Human	15
SAL_BA9648AA	Human	15
SAL_BA9878AA	Human	15
SAL_BA9910AA	Human	14
SAL_CA0073AA	Human	14
SAL_CA0117AA	Human	14
SAL_CA0294AA	Human	14
SAL_CA0811AA	Human	15
SAL_CA3099AA	Human	14
SAL_CA3621AA	Human	15
SAL_CA3717AA	Human	14
SAL_CA4021AA	Human	15
SAL_CA4334AA	Human	15
SAL_CA4443AA	Other Mammal	14
SAL_CA4551AA	Human	14
SAL_CA4610AA	Human	14
SAL_CA4855AA	Other Mammal	14
SAL_CA4892AA	Human	14
SAL_CA4959AA	Human	14
SAL_CA5041AA	Human	14
SAL_CA5095AA	Human	15
SAL_CA5213AA	Human	15
SAL_CA5289AA	Human	15
SAL_CA5512AA	Human	14
SAL_CA5939AA	Human	14
SAL_CA5979AA	Human	14
SAL_CA6377AA	Human	14
SAL_CA6458AA	Environment	14
SAL_DA0494AA	Human	12
SAL_DA0931AA	Human	12
SAL_DA1259AA	Human	12
SAL_EA7001AA	Human	ND
SAL_EA7588AA	Human	ND
SAL_EA7794AA	Human	ND
SAL_EA7892AA	Human	ND
SAL_FA4240AA	Human	15
SAL_FA4281AA	Human	15
SAL_FA4353AA	Food	15
SAL_FA4411AA	Food	15
SAL_FA4456AA	Human	15
SAL_FA4608AA	Human	15
SAL_IA3144AA	Human	15
SAL_IA4987AA	Livestock	17

SAL_IA4989AA	Livestock	17
SAL_IA7269AA	Human	17
SAL_IA7278AA	Livestock	17
SAL_IA7354AA	Livestock	17
SAL_JA0606AA	Retail Meat	6
SAL_JA6550AA	Human	17
SAL_JA8044AA	Human	17
SAL_JA8250AA	Human	17
SAL_KA4228AA	Canine	9
SAL_KA4235AA	Bovine	9
SAL_KA4237AA	Bovine	9
SAL_KA4244AA	Canine	9
SAL_KA4251AA	Bovine	9
SAL_KA4253AA	Canine	9
SAL_KA4260AA	Ovine	9
SAL_KA4267AA	Bovine	9
SAL_KA4269AA	Bovine	9
SAL_KA4275AA	Bovine	9
SAL_KA4282AA	Ovine	9
SAL_KA4284AA	Bovine	9
SAL_KA4291AA	Bovine	9
SAL_KA4298AA	Canine	9
SAL_KA4307AA	Bovine	9
SAL_KA4314AA	Bovine	9
SAL_LA2139AA	Bovine	6
SAL_LA2627AA	Ovine	7
SAL_LA2629AA	Bovine	7
SAL_LA2634AA	Bovine	7
SAL_LA2635AA	Bovine	8
SAL_LA2636AA	Bovine	8
SAL_LA2643AA	Bovine	8
SAL_LA2645AA	Bovine	7
SAL_LA2650AA	Bovine	7
SAL_LA2651AA	Bovine	8
SAL_LA2652AA	Bovine	8
SAL_LA2661AA	Bovine	6
SAL_LA2666AA	Bovine	7
SAL_LA2668AA	Bovine	7
SAL_LA2674AA	Bovine	9
SAL_LA2681AA	Bovine	7
SAL_LA2683AA	Bovine	7
SAL_LA2684AA	Human	1
SAL_LA2689AA	Bovine	8
SAL_LA2697AA	Bovine	7
SAL_LA2699AA	Ovine	7
SAL_LA2700AA	Human	1
SAL_LA2705AA	Bovine	8
SAL_LA2709AA	Bovine	4
SAL_LA2713AA	Bovine	7

SAL_LA2715AA	Bovine	7
SAL_LA2716AA	Human	4
SAL_MA1736AA	Food	15
SAL_MA1742AA	Human	16
SAL_MA1755AA	Food	15
SAL_MA1756AA	Human	17
SAL_MA1757AA	Human	15
SAL_MA1763AA	Human	17
SAL_MA1786AA	Human	17
SAL_MA1794AA	Food	17
SAL_MA1809AA	Food	17
SAL_MA1814AA	Human	16
SAL_MA1851AA	Human	16
SAL_MA1869AA	Human	16
SAL_MA1911AA	Human	17
SAL_MA1914AA	Human	17
SAL_MA1925AA	Human	16
SAL_MA1930AA	Human	17
SAL_MA1951AA	Food	17
SAL_MA1978AA	Human	16
SAL_MA1995AA	Human	16
SAL_MA1998AA	Human	17
SAL_MA2006AA	Human	15
SAL_MA2007AA	Human	17
SAL_MA2012AA	Food	15
SAL_MA2022AA	Food	17
SAL_MA2046AA	Human	16
SAL_MA2059AA	Human	15
SAL_MA2065AA	Human	15
SAL_MA2066AA	Human	16
SAL_MA2084AA	Human	16
SAL_MA2086AA	Human	15
SAL_MA2093AA	Human	17
SAL_MA2099AA	Human	16
SAL_MA2139AA	Food	17
SAL_MA2163AA	Food	17
SAL_MA2179AA	Human	17
SAL_MA2189AA	Human	16
SAL_MA2202AA	Human	16
SAL_MA2216AA	Human	17
SAL_MA2324AA	Human	16
SAL_MA2325AA	Human	16
SAL_MA2338AA	Human	16
SAL_MA2358AA	Human	15
SAL_MA2373AA	Human	16
SAL_MA2376AA	Environment	17
SAL_MA2406AA	Human	16
SAL_MA2437AA	Human	17
SAL_MA2443AA	Human	17

SAL_MA2445AA	Food	17
SAL_MA2455AA	Food	17
SAL_MA3953AA	Human	17
SAL_MA3967AA	Human	16
SAL_MA3971AA	Human	15
SAL_MA3976AA	Human	17
SAL_MA3985AA	Human	17
SAL_MA3990AA	Food	15
SAL_MA3993AA	Human	17
SAL_MA4008AA	Human	15
SAL_MA4026AA	Human	17
SAL_MA4028AA	Human	17
SAL_MA4424AA	Food	15
SAL_NA2034AA	Livestock	17
SAL_NA3147AA	Livestock	17
SAL_NA3153AA	Livestock	17
SAL_NA3155AA	Livestock	17
SAL_NA3160AA	Livestock	17
SAL_NA3161AA	Livestock	17
SAL_NA3163AA	Livestock	17
SAL_OA5919AA	Human	17
SAL_OA5921AA	Human	18
SAL_OA5922AA	Human	18
SAL_OA5947AA	Human	18
SAL_OA5948AA	Human	18
SAL_OA5949AA	Human	17
SAL_OA5950AA	Human	16
SAL_OA5951AA	Human	16
SAL_OA5952AA	Human	18
SAL_OA5953AA	Human	18
SAL_PA5611AA	Human	18
SAL_PA8667AA	Human	18
SAL_QA9792AA	Human	17
SAL_RA6165AA	Food	14
SAL_RA6654AA	Food	18
SAL_RA7296AA	Human	16
SAL_SA1476AA	Livestock	9
SAL_SA4879AA	Human	18
SAL_SA5970AA	Human	18
SAL_SA6480AA	Human	18
SAL_SA6550AA	Human	18
SAL_TA0328AA	Human	18
SAL_TA8487AA	Human	18
SAL_TA8964AA	Wild Animal	18
SAL_UA5440AA	Human	18
SAL_UA6527AA	Human	18
SAL_UA6992AA	Human	18
SAL_UA7022AA	Human	18
SAL_VA2205AA	Food	18

SAL_WA1688AA	Wild Animal	15
SAL_WA1967AA	Human	19
SAL_WA3455AA	Food	19
SAL_WA3458AA	Human	19
SAL_WA3469AA	Food	19
SAL_WA4487AA	Human	19
SAL_WA5156AA	Food	19
SAL_WA6581AA	Human	19
SAL_XA4061AA	Human	19
SAL_XA4635AA	Human	19
SAL_YA4506AA	Bovine	9
SAL_YA4507AA	Bovine	9
SAL_YA4508AA	Bovine	9
SAL_YA4510AA	Bovine	9
SAL_YA4511AA	Bovine	9
SAL_YA4527AA	Bovine	9
SAL_YA4529AA	Bovine	9
SAL_YA4530AA	Bovine	9
SAL_YA4534AA	Bovine	9
SAL_YA4758AA	Bovine	9
SAL_YA4761AA	Bovine	9
SAL_YA4852AA	Bovine	9
SAL_YA4934AA	Bovine	9
SAL_YA5007AA	Bovine	9
SAL_YA5008AA	Bovine	9
SAL_YA5010AA	Bovine	9
SAL_YA5014AA	Bovine	9
SAL_YA5016AA	Bovine	9
SAL_YA5146AA	Bovine	9
SAL_YA5149AA	Bovine	9
SAL_YA5207AA	Bovine	9
SAL_YA5270AA	Bovine	9
SAL_YA5340AA	Bovine	9
SAL_ZA0150AA	Human	19
SAL_ZA3315AA	Human	19
SAL_ZA7190AA	Bovine	9
SAL_ZA7191AA	Bovine	9
SAL_ZA7193AA	Bovine	9
SAL_ZA7201AA	Bovine	9
SAL_ZA7202AA	Bovine	9
SAL_ZA7203AA	Bovine	9
SAL_ZA7282AA	Bovine	9
SAL_ZA7283AA	Bovine	9
SAL_ZA7284AA	Bovine	9
SAL_ZA7285AA	Bovine	9
SAL_ZA7393AA	Bovine	9
SAL_ZA7394AA	Bovine	9
SAL_ZA7395AA	Bovine	9
SAL_ZA7396AA	Bovine	9

SAL_ZA7471AA	Bovine	9
SAL_ZA7472AA	Bovine	9
SAL_ZA7473AA	Bovine	9
SAL_ZA7474AA	Bovine	9
SAL_ZA7475AA	Bovine	9
SAL_ZA8220AA	Human	19
SAL_ZA8453AA	Human	19
SAL_ZA8476AA	Human	19
SAL_ZA9888AA	Human	19

ND – No date available

Table 9.5 – Metadata of 266 *S.* Typhimurium isolates downloaded from Enterobase and used in comparative analysis.

Barcode	Source	Collection year
SAL_AB0987AA	Food	2019
SAL_AB1034AA	Food	2019
SAL_BA5709AA	Livestock	2002
SAL_BA5727AA	Human	2004
SAL_BA5729AA	Livestock	2003
SAL_BA5742AA	Livestock	2004
SAL_BA5746AA	Livestock	2003
SAL_BA6099AA	Livestock	1996
SAL_BA6101AA	Livestock	1996
SAL_BA6103AA	Livestock	1996
SAL_BA6105AA	Livestock	1996
SAL_BA6107AA	Livestock	1996
SAL_BA6113AA	Livestock	1995
SAL_BA6115AA	Livestock	1995
SAL_BA6118AA	Livestock	1995
SAL_BA6120AA	Livestock	1995
SAL_BA6123AA	Livestock	1995
SAL_BA6125AA	Livestock	1995
SAL_BA6127AA	Livestock	1995
SAL_BA6129AA	Livestock	1995
SAL_BA6176AA	Human	2001
SAL_BA6271AA	Livestock	2004
SAL_BA6279AA	Livestock	1999
SAL_BA6280AA	Livestock	1999
SAL_BA6283AA	Livestock	1998
SAL_BA6287AA	Livestock	1997
SAL_BA6288AA	Livestock	1997
SAL_BA6290AA	Livestock	1997
SAL_BA6292AA	Livestock	1997
SAL_BA7582AA	Human	2015
SAL_BA8171AA	Human	2014
SAL_BA8234AA	Human	2014
SAL_BA8253AA	Livestock	2014
SAL_BA8835AA	Human	2014
SAL_BA8972AA	Human	2015
SAL_BA9417AA	Human	2014
SAL_BA9577AA	Human	2014
SAL_BB1330AA	Livestock	2008
SAL_BB9057AA	Human	2020
SAL_BB9065AA	Human	2020
SAL_BB9066AA	Human	2020
SAL_BB9069AA	Livestock	2020
SAL_CA0341AA	Human	2001
SAL_CA0384AA	Livestock	2010
SAL_CA0388AA	Livestock	2009

SAL_CA0390AA	Livestock	2008
SAL_CA0394AA	Livestock	2008
SAL_CA0398AA	Livestock	2006
SAL_CA0400AA	Livestock	2006
SAL_CA0402AA	Livestock	2005
SAL_CA0404AA	Livestock	2005
SAL_CA0406AA	Livestock	2005
SAL_CA0893AA	Food	2015
SAL_CA1141AA	Human	2014
SAL_CA1467AA	Food	2015
SAL_CA1528AA	Food	2015
SAL_CA1627AA	Human	2014
SAL_CA2294AA	Human	2014
SAL_CA2349AA	Human	2014
SAL_CA2350AA	Human	2014
SAL_CA2857AA	Human	2014
SAL_CA2864AA	Human	2014
SAL_CA2944AA	Food	2015
SAL_CA3100AA	Food	2015
SAL_CA3188AA	Food	2015
SAL_CA3451AA	Human	2014
SAL_CA3523AA	Human	2014
SAL_CA3885AA	Human	2015
SAL_CA3940AA	Human	2015
SAL_CA3944AA	Human	2014
SAL_CA4145AA	Human	2015
SAL_CA4880AA	Human	2014
SAL_CA4897AA	Human	2014
SAL_CA5622AA	Food	2015
SAL_CA6029AA	Livestock	2014
SAL_CB0154AA	Human	2020
SAL_CB3316AA	Food	2019
SAL_CB6517AA	Wild Animal	2014
SAL_DA0384AA	Human	2012
SAL_DA0405AA	Human	2012
SAL_DA0469AA	Human	2012
SAL_DA0721AA	Food	2012
SAL_DA4587AA	Livestock	1997
SAL_DA4605AA	Livestock	1998
SAL_DA4606AA	Livestock	1998
SAL_DA4607AA	Livestock	1998
SAL_DA4608AA	Livestock	1999
SAL_DA4611AA	Livestock	1998
SAL_DA4621AA	Livestock	1997
SAL_DA4641AA	Livestock	2003
SAL_DA4642AA	Livestock	1995
SAL_DA4654AA	Livestock	1997
SAL_DA4658AA	Livestock	1996

SAL_DA4659AA	Livestock	1998
SAL_DA4665AA	Livestock	1996
SAL_DA4666AA	Livestock	1994
SAL_DA4668AA	Livestock	1995
SAL_DA4677AA	Livestock	1996
SAL_DA4680AA	Livestock	1994
SAL_DA4681AA	Livestock	1997
SAL_DA4682AA	Livestock	2001
SAL_DA4684AA	Livestock	1996
SAL_DA4685AA	Livestock	1996
SAL_DA4686AA	Livestock	1995
SAL_DA4688AA	Livestock	2004
SAL_DA4690AA	Livestock	1995
SAL_DA4691AA	Livestock	1999
SAL_DA4704AA	Livestock	1994
SAL_DA4705AA	Livestock	1996
SAL_DA4706AA	Livestock	1996
SAL_DA4707AA	Livestock	1997
SAL_DA4713AA	Livestock	1994
SAL_DB7819AA	Livestock	2020
SAL_DB8011AA	Livestock	2020
SAL_FA1760AA	Livestock	2003
SAL_FA1811AA	Livestock	2001
SAL_FA3458AA	Human	2015
SAL_FA3459AA	Human	2015
SAL_FA3464AA	Human	2015
SAL_FA3488AA	Human	2015
SAL_FA3506AA	Human	2015
SAL_FA3511AA	Human	2015
SAL_FA3513AA	Human	2015
SAL_FA3526AA	Human	2015
SAL_FA3549AA	Human	2015
SAL_FA3567AA	Human	2015
SAL_FA3569AA	Human	2015
SAL_FA3586AA	Human	2015
SAL_FA3624AA	Human	2015
SAL_FA3637AA	Human	2015
SAL_FA3718AA	Environment	2015
SAL_FA3822AA	Human	2015
SAL_FA3833AA	Human	2016
SAL_FA3969AA	Human	2015
SAL_FA4107AA	Human	2015
SAL_FA4113AA	Human	2016
SAL_FA4143AA	Human	2015
SAL_FA4278AA	Human	2016
SAL_FA4543AA	Livestock	2015
SAL_IA1271AA	Human	2016
SAL_IA1876AA	Livestock	2003

SAL_IA2792AA	Human	2016
SAL_IA2848AA	Human	2016
SAL_IA4988AA	Human	2017
SAL_IA7281AA	Human	2017
SAL_IA9072AA	Human	2016
SAL_JA2247AA	Livestock	2015
SAL_JA4775AA	Livestock	2017
SAL_JA5406AA	Human	2017
SAL_JA5407AA	Human	2016
SAL_JA5545AA	Human	2017
SAL_JA5553AA	Human	2017
SAL_JA5770AA	Human	2017
SAL_JA6834AA	Human	2017
SAL_JA7206AA	Livestock	2017
SAL_JA7219AA	Livestock	2017
SAL_JA7876AA	Human	2017
SAL_JA8244AA	Human	2016
SAL_JA8263AA	Human	2017
SAL_JA8335AA	Human	2016
SAL_JA8385AA	Human	2017
SAL_KA3818AA	Livestock	2009
SAL_KA7177AA	Livestock	2017
SAL_LA5256AA	Wild Animal	2014
SAL_LA6929AA	Livestock	2017
SAL_LA7591AA	Human	2016
SAL_LA7598AA	Human	2016
SAL_LA7923AA	Human	2016
SAL_LA7925AA	Human	2016
SAL_LA7933AA	Human	2016
SAL_NA2038AA	Human	2017
SAL_NA2042AA	Human	2017
SAL_NA2056AA	Livestock	2017
SAL_NA2059AA	Human	2017
SAL_NA4411AA	Human	2017
SAL_NA4416AA	Human	2017
SAL_NA8042AA	Food	2017
SAL_NA8057AA	Food	2017
SAL_NA8061AA	Food	2017
SAL_NA8280AA	Food	2017
SAL_PA4571AA	Human	2017
SAL_PA4976AA	Human	2017
SAL_PA5585AA	Human	2017
SAL_PA5589AA	Human	2016
SAL_PA5590AA	Human	2016
SAL_PA6113AA	Human	2017
SAL_PA6116AA	Human	2016
SAL_PA6119AA	Human	2016
SAL_PA6367AA	Human	2016

SAL_PA6368AA	Human	2016
SAL_PA6391AA	Human	2016
SAL_PA6560AA	Human	2018
SAL_PA7261AA	Human	2018
SAL_PA7716AA	Human	2017
SAL_PA7735AA	Human	2017
SAL_PA8746AA	Human	2018
SAL_QA1346AA	Human	2018
SAL_QA2646AA	Human	2018
SAL_QA2683AA	Human	2018
SAL_QA3333AA	Human	2018
SAL_QA4756AA	Human	2018
SAL_QA6163AA	Human	2018
SAL_QA6722AA	Human	2018
SAL_QA6723AA	Human	2018
SAL_QA7382AA	Human	2018
SAL_QA7663AA	Human	2018
SAL_QA7664AA	Human	2018
SAL_QA7670AA	Human	2018
SAL_QA7673AA	Human	2018
SAL_QA7741AA	Human	2018
SAL_QA7758AA	Human	2018
SAL_QA7780AA	Human	2018
SAL_QA7782AA	Human	2018
SAL_QA8660AA	Human	2018
SAL_QA8801AA	Human	2018
SAL_QA9369AA	Human	2017
SAL_QA9421AA	Human	2018
SAL_QA9427AA	Human	2017
SAL_RA0279AA	Human	2018
SAL_RA0365AA	Human	2018
SAL_RA0702AA	Human	2018
SAL_RA0771AA	Human	2018
SAL_RA0825AA	Human	2018
SAL_RA1024AA	Human	2018
SAL_RA3771AA	Human	2018
SAL_RA4279AA	Food	2017
SAL_RA4311AA	Food	2017
SAL_RA4371AA	Food	2018
SAL_RA4433AA	Wild Animal	2018
SAL_RA5439AA	Environment	2017
SAL_RA6593AA	Human	2016
SAL_RA6595AA	Human	2016
SAL_RA6714AA	Food	2014
SAL_RA7008AA	Human	2016
SAL_UA2022AA	Livestock	2014
SAL_UA2034AA	Wild Animal	2014
SAL_UA2052AA	Livestock	2016

SAL_UA2094AA	Livestock	2016
SAL_UA2095AA	Livestock	2016
SAL_UA2101AA	Ovine/Goat	2016
SAL_UA2104AA	Ovine/Goat	2016
SAL_UA2105AA	Livestock	2016
SAL_UA2108AA	Ovine/Goat	2016
SAL_UA2124AA	Livestock	2016
SAL_UA2139AA	Livestock	2014
SAL_UA2143AA	Livestock	2016
SAL_UA2151AA	Livestock	2014
SAL_UA2156AA	Livestock	2016
SAL_VA2055AA	Food	2017
SAL_VA2223AA	Food	2018
SAL_VA4397AA	Food	2017
SAL_YA4627AA	Companion Animal	2009
SAL_YA4851AA	Livestock	2008
SAL_YA4919AA	Livestock	2009
SAL_YA4956AA	Livestock	2008
SAL_YA4997AA	Livestock	2008
SAL_YA5000AA	Livestock	2008
SAL_YA5036AA	Livestock	2008
SAL_YA5063AA	Companion Animal	2009
SAL_YA5173AA	Ovine/Goat	2009
SAL_YA5265AA	Livestock	2009
SAL_YA5275AA	Livestock	2008
SAL_YA5276AA	Companion Animal	2008
SAL_ZA7366AA	Livestock	2009
SAL_ZA7407AA	Companion Animal	2008
SAL_ZA7528AA	Livestock	2008

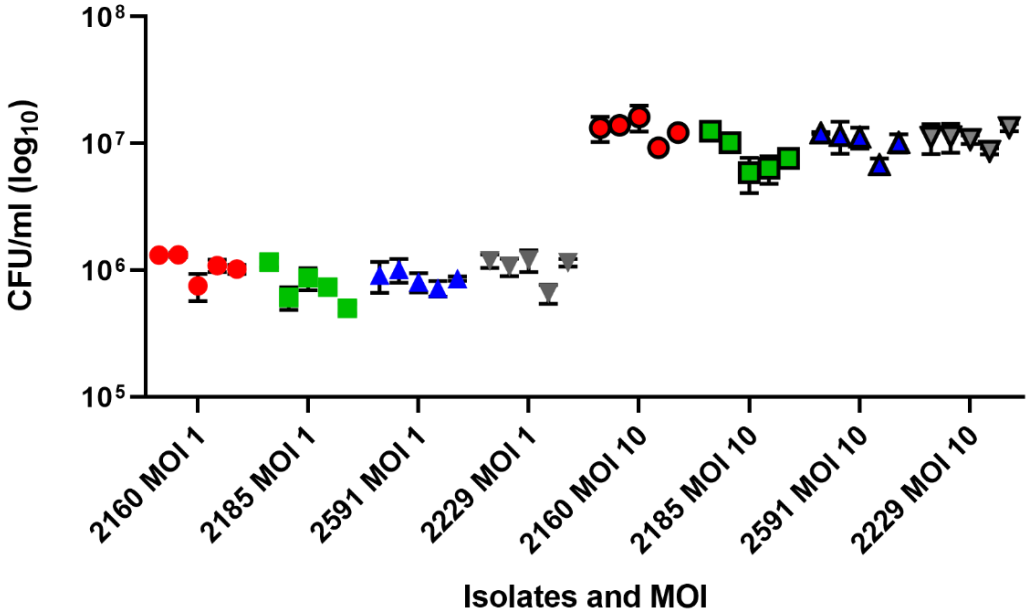


Figure 9.10 – *S.* Dublin inocula used to infect BCECs at MOIs of 1 and 10. *S.* Dublin isolates were grown into log phase and diluted in bovine caruncular epithelial cell (BCEC) medium and used to inoculate BCECs to a multiplicity of infection (MOI) of 1 and 10. Inocula were assessed for accuracy by taking a sample of the media which was serially diluted and plated on nutrient agar in accordance with the Miles and Misra method (Miles, Misra, and Irwin, 1938), and cultured overnight at 37°C until colonies could be counted. n=5

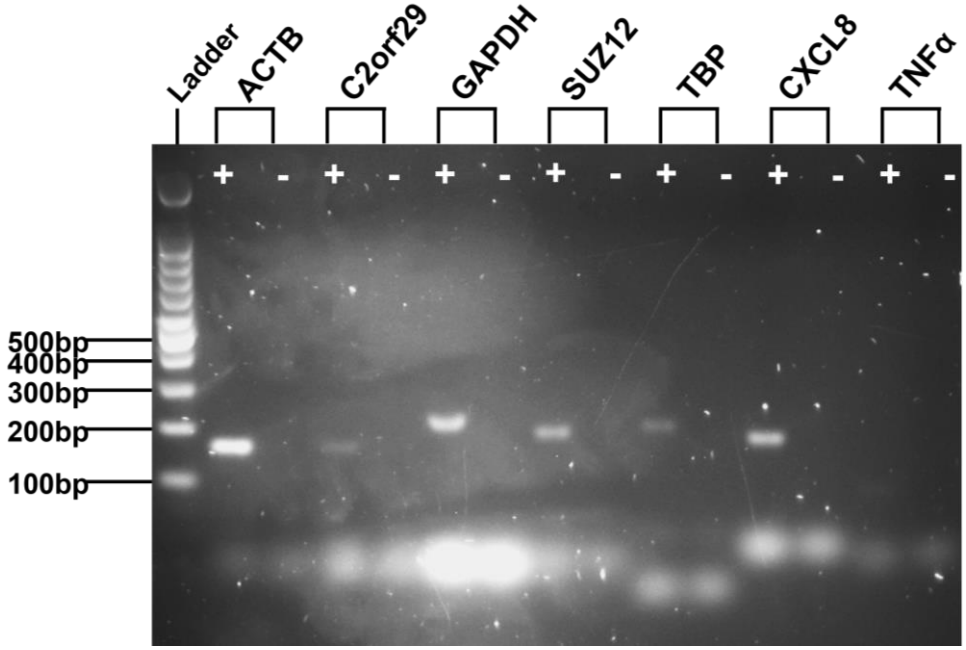


Figure 9.11 – 2% agarose gel of PCR products with different qPCR primer pairs used to discern the host response of BCECs to *S.* Dublin infection. Gel was run for 1h 40m at 90V. "+" and "-" denotes wells with and without template DNA respectively. The band for TNFα is weak but present.

Table 9.6 – Linear regression of standards for ACTB from each experimental set of BCECs stimulated with *S.* Dublin. Standard dilutions of cDNA used were 1/10, 1/100, 1/1,000, 1/10,000 and 1/100,000 to create a standard curve upon which a linear regression was performed to calculate the corresponding efficiency. To be deemed appropriate for use by the MIQE guidelines, the R^2 value needed to be above 98%, the slope value needed to be between -3.10 and -3.58, and the efficiency needed to be between 90% and 110% (Bustin et al., 2009).

Experiment repeat	R^2 Value	Slope Value	Corresponding efficiency
A	99.93%	-3.474	94.02%
B	99.58%	-3.358	98.52%
C	99.44%	-3.419	96.10%
D	99.58%	-3.375	97.83%
E	99.75%	-3.567	90.70%

Table 9.7 – Linear regression of standards for C2orf29 from each experimental set of BCECs stimulated with *S.* Dublin. Standard dilutions of cDNA used were 1/3, 1/10, 1/30, 1/100, 1/300 and 1/1,000 to create a standard curve upon which a linear regression was performed to calculate the corresponding efficiency. To be deemed appropriate for use by the MIQE guidelines, the R^2 value needed to be above 98%, the slope value needed to be between -3.10 and -3.58, and the efficiency needed to be between 90% and 110% (Bustin et al., 2009).

Experiment repeat	R^2 Value	Slope Value	Corresponding efficiency
A	98.39%	-3.443	95.18%
B	98.70%	-3.174	106.57%
C[a]	98.06%	-3.231	103.94%
D[b]	98.92%	-3.539	91.68%
E	98.37%	-3.190	105.82%

a – removed 1/30 dilution from analysis

b – removed one value from 1/100 dilution as an outlier

Table 9.8 – Linear regression of standards for CXCL8 from each experimental set of BCECs stimulated with *S.* Dublin. Standard dilutions of cDNA used were 1/3, 1/10, 1/30, 1/100, 1/300 and 1/1,000 to create a standard curve upon which a linear regression was performed to calculate the corresponding efficiency. To be deemed appropriate for use by the MIQE guidelines, the R^2 value needed to be above 98%, the slope value needed to be between -3.10 and -3.58, and the efficiency needed to be between 90% and 110% (Bustin et al., 2009).

Experiment repeat	R^2 Value	Slope Value	Corresponding efficiency
A	99.24%	-3.173	106.61%
B	99.19%	-3.297	101.05%
C	99.13%	-3.431	95.64%
D	99.55%	-3.231	103.94%
E	99.65%	-3.164	107.04%

Table 9.9 - Linear regression of standards for TNFα from each experimental set of BCECs stimulated with *S.* Dublin. Standard dilutions of cDNA used were 1/3, 1/10, 1/30, 1/100, 1/300 and 1/1,000 to create a standard curve upon which a linear regression was performed to calculate the corresponding efficiency. To be deemed appropriate for use by the MIQE guidelines, the R^2 value needed to be above 98%, the slope value needed to be between -3.10 and -3.58, and the efficiency needed to be between 90% and 110% (Bustin et al., 2009). Experimental set D was not used in the analysis as R^2 and Slope values were outside of the acceptable limits according to the MIQE guidelines.

Experiment repeat	R^2 Value	Slope Value	Corresponding efficiency
A[a]	98.37%	-3.494	93.29%
B[b]	98.51%	-3.132	108.59%
C[a]	98.42%	-3.123	109.03%
D - excluded	95.51%	-4.063	76.25%
E	98.14%	-3.249	103.14%

[a]1/300 dilution was removed as outlying standard

[b]1/100 dilution as a whole and one data point of 1/30 dilution were removed as outliers

NA = standards were not appropriate according to the MIQE guidelines, so samples were not used in downstream data analysis, n=3

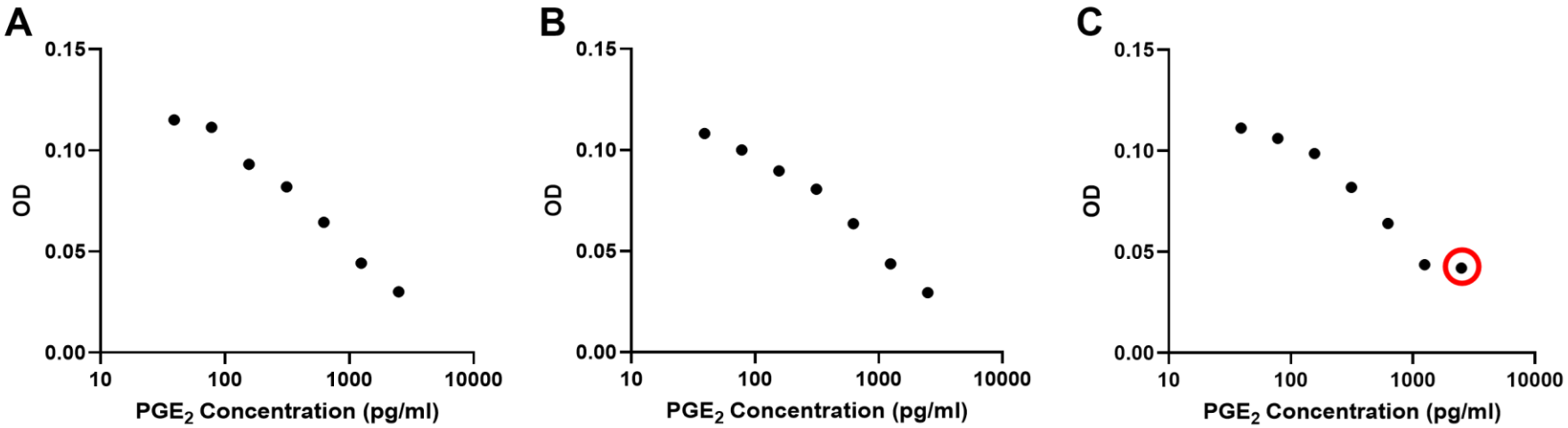


Figure 9.12 – Standard curves generated during Prostaglandin E_2 (PGE_2) Enzyme-Linked Immunosorbent Assays (ELISAs) for supernatant samples taken from BCECs stimulated with *S.* Dublin. Bovine caruncular epithelial cells (BCECs) were infected with *S.* Dublin isolates at multiplicities of infection (MOI) of 1 and 10, heat-killed *S.* Dublin or *S.* Typhimurium lipopolysaccharide (LPS). Three different standard curves were generated in the process of measuring the concentration of all samples for PGE_2 production. One outlier (red circle, C) was removed from analysis and replaced with the average values of the corresponding standards from the previous two analyses (A and B). These curves were used to extrapolate two points above and below the original standards and the new standard curves generated from this information were used to extrapolate the concentrations of PGE_2 produced in the samples.

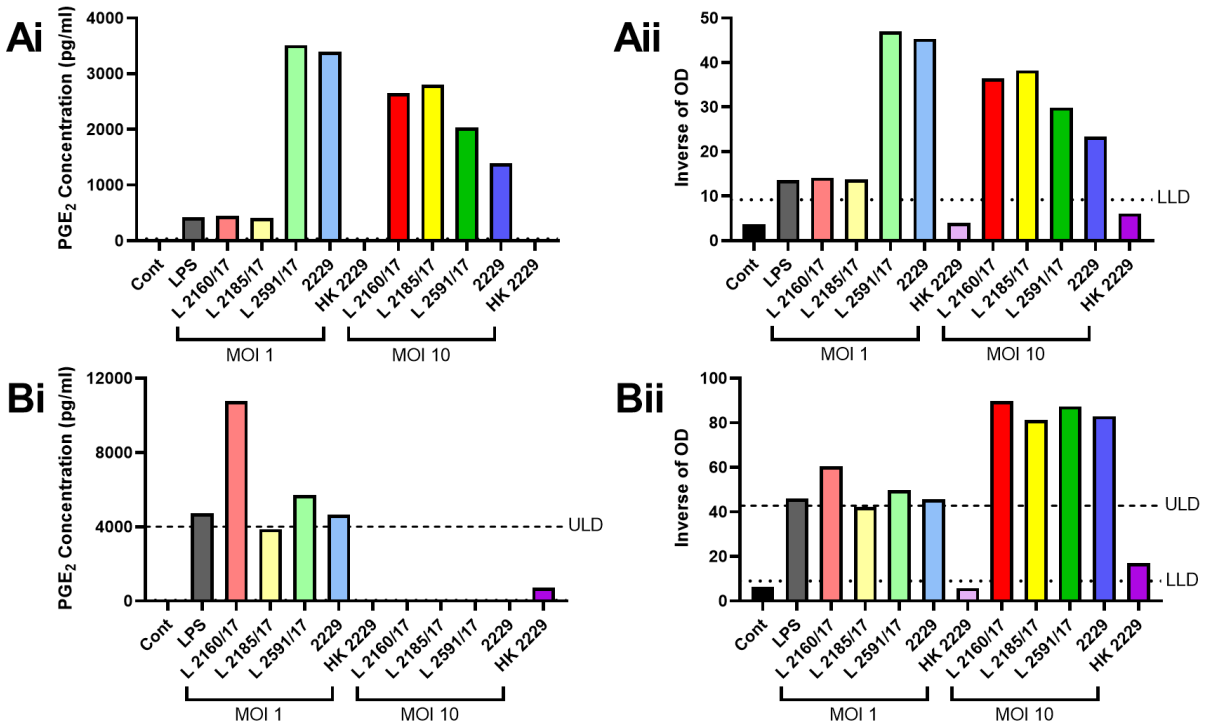


Figure 9.13 – Concentrations of PGE_2 produced by BCECs infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or LPS after 24h, measured by competitive ELISA. Five infection/stimulation experiments (A-E) were conducted using Bovine Caruncular Epithelial Cells (BCECs) and *S.* Dublin isolates at multiplicities of infection (MOIs) of 1 and 10. After 24h of infection/stimulation, the supernatants were removed and stored at -80°C until use. Competitive Enzyme linked immunosorbent assays (ELISAs) were conducted to quantify the concentration of PGE_2 produced by the BCECs in response to the bacteria or stimulation regimen. ELISA results from experimental sets A (Ai and Aii) and B (Bi and Bii) which includes concentrations (Ai and Bi) and inverse optical density (OD) readings (Aii and Bii). ULD denotes the upper limit of detection as defined by the ELISA used, whilst LLD denotes the lower limit of detection dictated by the standards in each experimental set.

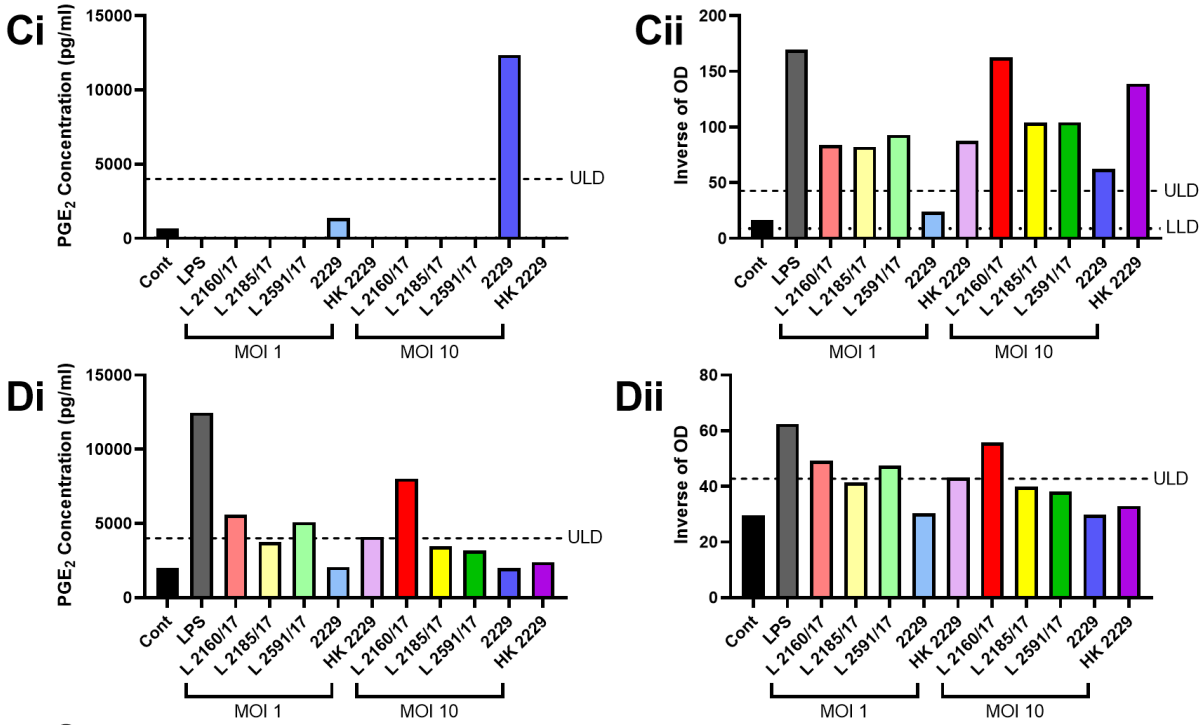


Figure 9.14 - Concentrations of PGE_2 produced by BCECs infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or LPS after 24h, measured by competitive ELISA. Five infection/stimulation experiments (A-E) were conducted using Bovine Caruncular Epithelial Cells (BCECs) and *S.* Dublin isolates at multiplicities of infection (MOIs) of 1 and 10. After 24h of infection/stimulation, the supernatants were removed and stored at -80°C until use. Competitive Enzyme linked immunosorbent assays (ELISAs) were conducted to quantify the concentration of PGE_2 produced by the BCECs in response to the bacteria or stimulation regimen. ELISA results from experimental sets C (Ci and Cii) and D (Di and Dii) which includes concentrations (Ci and Di) and inverse optical density (OD) readings (Cii and Dii). ULD denotes the upper limit of detection as defined by the ELISA used, whilst LLD denotes the lower limit of detection dictated by the standards in each experimental set.

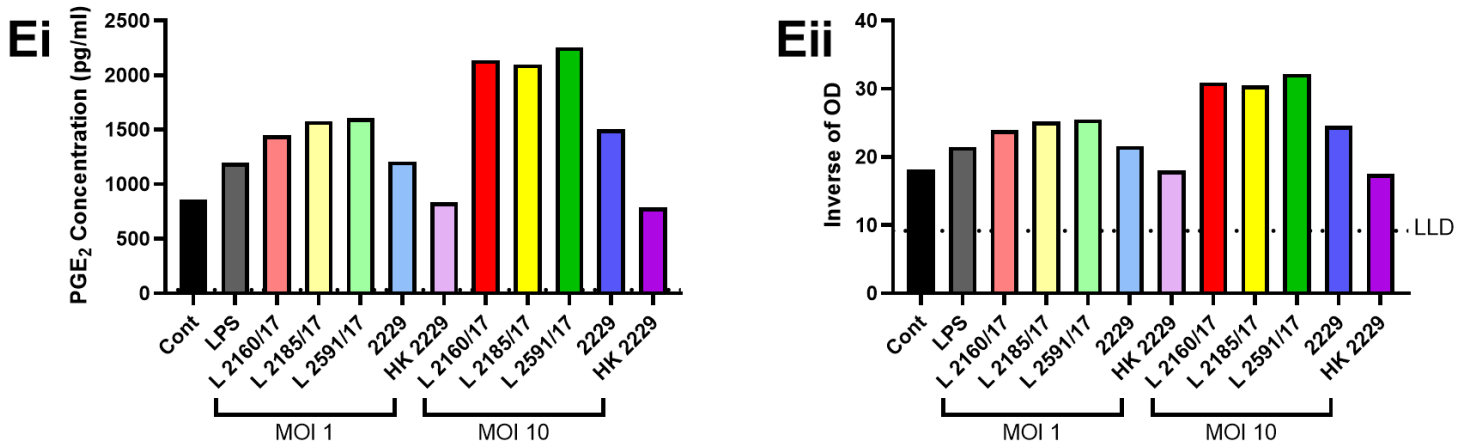


Figure 9.15 - Concentrations of PGE_2 produced by BCECs infected with *S.* Dublin isolates or stimulated with heat-killed *S.* Dublin or LPS after 24h, measured by competitive ELISA. Five infection/stimulation experiments (A-E) were conducted using Bovine Caruncular Epithelial Cells (BCECs) and *S.* Dublin isolates at multiplicities of infection (MOIs) of 1 and 10. After 24h of infection/stimulation, the supernatants were removed and stored at -80°C until use. Competitive Enzyme linked immunosorbent assays (ELISAs) were conducted to quantify the concentration of PGE_2 produced by the BCECs in response to the bacteria or stimulation regimen. ELISA results from experimental set E which includes concentrations (Ei) and inverse optical density (OD) readings (Eii). ULD denotes the upper limit of detection as defined by the ELISA used, whilst LLD denotes the lower limit of detection dictated by the standards in each experimental set.

Table 9.10 - Antimicrobial resistance genes identified in *S.* Typhimurium isolates, grouped into antibiotics, antibiotic classes and multi-drug compartments. The whole genome sequences of 266 *S.* Typhimurium isolates from various origins of isolation were compared to the MEGARes database containing all known AMR genes, with an identity threshold of 90.0% (Doster et al., 2020). Genes were grouped into antimicrobial glasses using information from CARD (Alcock et al., 2020).

Antimicrobial resistance	Total no. associated AMR genes in *S.* Typhimurium	AMR Genes	MEGARes Reference
Rifampin	1	*arr*	https://megares.meglab.org/browse/Rifampin/Rifampin_ADP-ribosyltransferase_Arr/ARR/
Bacitracin	1	*bacA*	https://megares.meglab.org/browse/Bacitracin/Undecaprenyl_pyrophosphate_phosphatase/BACA/
Chloramphenicol	2	*catA*	https://megares.meglab.org/browse/Phenicol/Chloramphenicol_acetyltransferases/CATA/
		floR	https://megares.meglab.org/browse/Phenicol/Phenicol_resistance_MFS_efflux_pumps/FLOR/
Fluoroquinolone	2	*qnrB*	https://megares.meglab.org/browse/Fluoroquinolones/Quinolone_resistance_protein_Qnr/QNRB/
		qnrS	https://megares.meglab.org/browse/Fluoroquinolones/Quinolone_resistance_protein_Qnr/QNRS/
Trimethoprim	1	*dfrA*	https://megares.meglab.org/browse/Trimethoprim/Dihydrofolate_reductase/DFRA/
Aminoglycosides	11	AAC3	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_N-acetyltransferases/AAC3/
		AAC6-PRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_N-acetyltransferases/AAC6-PRIME/
		acrD	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/ACRD/
		ANT3-DPRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-nucleotidyltransferases/ANT3-DPRIME/
		ANT3-DPRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-nucleotidyltransferases/ANT3-DPRIME/
		APH3-DPRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-phosphotransferases/APH3-DPRIME/
		APH3-DPRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-phosphotransferases/APH3-DPRIME/
		APH3-PRIME	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-phosphotransferases/APH3-PRIME/
		APH4	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-phosphotransferases/APH4/

		APH6	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_O-phosphotransferases/APH6/
		kdpE	https://megares.meglab.org/browse/Aminoglycosides/Aminoglycoside_efflux_pumps/KDPE/
Beta-lactam	7	*ampH*	https://megares.meglab.org/browse/betalactams/Penicillin_binding_protein/AMPH/
		carB	https://megares.meglab.org/browse/betalactams/Class_A_betalactamases/CARB/
		cmy	https://megares.meglab.org/browse/betalactams/Class_C_betalactamases/CMY/
		ctx	https://megares.meglab.org/browse/betalactams/Class_A_betalactamases/CTX/
		ctx	https://megares.meglab.org/browse/betalactams/Class_A_betalactamases/CTX/
		PBP2	https://megares.meglab.org/browse/betalactams/Penicillin_binding_protein/PBP2/
		tem	https://megares.meglab.org/browse/betalactams/Class_A_betalactamases/TEM/
Sulphonamides	3	*sulI*	https://megares.meglab.org/browse/Sulfonamides/Sulfonamide-resistant_dihydropteroate_synthases/SULI/
		sulII	https://megares.meglab.org/browse/Sulfonamides/Sulfonamide-resistant_dihydropteroate_synthases/SULII/
		sulIII	https://megares.meglab.org/browse/Sulfonamides/Sulfonamide-resistant_dihydropteroate_synthases/SULIII/
Tetracyclines	6	*tetA*	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_MFS_efflux_pumps/TETA/
		tetB	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_MFS_efflux_pumps/TETB/
		tetC	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_MFS_efflux_pumps/TETC/
		tetD	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_MFS_efflux_pumps/TETD/
		tetG	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_MFS_efflux_pumps/TETG/
		tetM	https://megares.meglab.org/browse/Tetracyclines/Tetracycline_resistance_ribosomal_protection_proteins/TETM/
Multi-drug	5	H-NS	https://megares.meglab.org/browse/Multi-drug_resistance/Multi-drug_RND_efflux_pumps/HNS/
		msbA	https://megares.meglab.org/browse/Multi-drug_resistance/Multi-drug_ABC_efflux_pumps/MSBA/
		oqxA	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_pumps/OQXA/
		oqxB	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_pumps/OQXB/

		sdiA	https://megares.meglab.org/browse/Multi-drug_resistance/Multi-drug_RND_efflux_pumps/SDIA/
MLS	3	*mefB*	https://megares.meglab.org/browse/MLS/MLS_resistance_MFS_efflux_pumps/MEFB/
		mphA	https://megares.meglab.org/browse/MLS/Macrolide_phosphotransferases/MPHA/
		mphB	https://megares.meglab.org/browse/MLS/Macrolide_phosphotransferases/MPHB/
Drug and biocide	15	*acrA*	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_pumps/ACRA/
		acrB	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_pumps/ACRB/
		bcr	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MFS_efflux_pumps/BCR/
		cmlA	https://megares.meglab.org/browse/Phenicol/Phenicol_resistance_MFS_efflux_pumps/CMLA/
		cpxAR	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_regulator/CPXAR/
		cpxAR	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_regulator/CPXAR/
		crp	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_regulator/CRP/
		emrA	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MFS_efflux_pumps/EMRA/
		emrB	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MFS_efflux_pumps/EMRB/
		emrD	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MFS_efflux_pumps/EMRD/
		emrR	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MFS_efflux_regulator/EMRR/
		marA	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_regulator/MARA/
		marR	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_RND_efflux_regulator/MARR/
		qacL	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_SMR_efflux_pumps/QACL/
		YOGI	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_ABC_efflux_pumps/YOGI/
Drug and metal	4	*mdtK*	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MATE_efflux_pumps/MDTK/

		mdtK	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MATE_efflux_pumps/MDTK/
		mdtK	https://megares.meglab.org/browse/Drug_and_biocide_resistance/Drug_and_biocide_MATE_efflux_pumps/MDTK/
		pmrG	https://megares.meglab.org/browse/Drug_and_metal_resistance/Drug_and_metal_MFS_efflux_pumps/PMRG/
Drug, biocide and metal	10	*baeR*	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_regulator/BAER/
		baeS	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_regulator/BAES/
		gesA	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/GESA/
		gesB	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/GESB/
		gesC	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/GESC/
		mdtA	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/MDTA/
		mdtB	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/MDTB/
		mdtC	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/MDTC/
		robA	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_RND_efflux_pumps/ROBA/
		soxS	https://megares.meglab.org/browse/Drug_and_biocide_and_metal_resistance/Drug_and_biocide_and_metal_resistance_regulator/SOXS/

Chapter 10 - PIP Reflective Statement

10.1 - Note to examiners

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

10.1.1 - Where you went on placement and the project you worked on

I undertook my 3 month placement at the Agriculture and Horticulture Development Board (AHDB) in Coventry. AHDB is a levy board funded by farmers from the dairy, beef and lamb, pork, potatoes, horticulture and cereals and oilseed sectors. I worked with the livestock sectors on the process of converting their downloadable PDF content into website format.

Like many businesses, AHDB increasingly communicates with its audience (their levy payers) through digital means. However, much of the information produced by AHDB in their "Knowledge Library" is in PDF format which is often overlooked by Google and other search engines. My project was part of a company-wide initiative to move PDF content into html pages as part of Search Engine Optimisation (SEO). SEO ultimately aims to have specific websites or businesses consistently returned in the top few search results.

I first mapped all of the content from the Dairy and Beef and Lamb sectors into spreadsheets and assigned categories to each document based on "process maps" produced by Knowledge Transfer (KT) and Marcomms teams. I then built spreadsheets to track the documents based on these process maps which required me to be very familiar with the content.

The process of producing content for levy payers is complex and involves several departments all contributing different aspects to the content. We therefore decided that it would be best to have a pilot trial with lameness in dairy cows as our topic of choice. I split down large, complex documents into smaller subsections and began to re-write the content in a more user- and web-friendly format using the skills I had learned from training provided by AHDB.

Aside from my project, I was able to help with an ongoing biosecurity campaign around lorry washing in the pig industry.

10.1.2 - The outcomes of your placement project

During the pilot trial of lameness in dairy cows, I contributed to the creation of a number of templates, spreadsheets tracking content and standard operating procedures which are now being used by other sectors in AHDB. My work is being used to train other members of staff in the process of content recreation. As well as helping to shape this process of content recreation, I was able to help write 30 web pages that have been uploaded onto the AHDB website. These web pages will help to support farmers seeking advice on managing lameness in their dairy herd.

Aside from my project, I was asked to write a short article for AHDB on Swine Dysentery in the UK which was published in the April edition of Pig World, the UKs leading pig magazine and the official magazine of the National Pig Association. My work will also contribute to a CPD module which will be included in the Red Tractor Standards for hauliers. The aim of the CPD module is to increase knowledge of how

to effectively wash out lorries to prevent the spread of disease, and will be used by hauliers and Red Tractor Assessors.

Following the completion of my placement, I was encouraged to stay working for AHDB on a voluntary basis because I had made a significant contribution to their work. Due to the COVID-19 pandemic disrupting lab work and subsequently allowing me to work more flexibly around my PhD, I was able to oblige. This was a voluntary role for up to 15 hours per week (in line with DTP regulations and guidance), and after a few weeks of working, my line manager expressed the company's desire to begin to pay me for my time on a zero hours contract, again in line with DTP regulations.

Perhaps the most important outcome from my placement has been an increase in my self-confidence and confidence in my work. I was very nervous to go on placement because I often feel my work is not good enough and that I will let people down. However, all of the feedback I received from line managers and people I worked with has been positive which has been a huge boost.

10.1.3 - What skills you developed whilst on placement

My writing skills were significantly developed whilst I was on placement. The style of writing for the web is very different to the academic style that I am used to, and I was formally trained on this as well as being able to write 30 pages of content. The article on swine dysentery in the UK was a slightly different style of writing again and it was very useful to get feedback on all of my work.

Due to the disruptions I experienced during my placement, first with the fire at AHDB headquarters and subsequently with the COVID-19 disruptions, I inadvertently developed my resilience and ability to cope with difficult scenarios and uncertainty. Staff at AHDB were encouraged to also take training modules in stress management, resilience and self-management which were useful when dealing with these stressful situations. My ability to manage my own and others

expectations when it comes to setting deadlines has improved, as well as my time management skills generally.

10.1.4 - Any impact your placement has had on your career plans

My placement with AHDB has greatly impacted my career plans. Working for AHDB has solidified my desire to work around agriculture and livestock, be that thorough research or in a company like AHDB. I will have much more confidence in myself when I apply for jobs, and I know that my placement will have significantly improved my career prospects because I now have relevant experience outside of my undergraduate degree and PhD. I feel more confident pursuing a career outside of academia following my placement.