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Exploring the biological basis for *Salmonella* persistence in food manufacturing environments

Amreen Bashir

Doctor of philosophy

ASTON UNIVERSITY

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microarray
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scanning
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Phenotypically
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clinical
biolog
microscopy
Laboratory
establish
genotype
panel
persists
representative
resident
identify
survival
genes
harbour
rate
relative
strains

THE UNIVERSITY OF ASTON IN BIRMINGHAM

Exploring the biological basis for *Salmonella* persistence in food manufacturing environments

A thesis submitted by Amreen Bashir for the degree of Doctor of Philosophy

2015

The persistence of *Salmonella* spp. in low moisture foods is a challenge for the food industry as despite control strategies already in place, notable outbreaks still occur. The aim of this study was to characterise isolates of *Salmonella*, known to be persistent in the food manufacturing environment, by comparing their microbiological characteristics with a panel of matched clinical and veterinary isolates. The gross morphology of the challenge panel was phenotypically characterised in terms of cellular size, shape and motility. In all the parameters measured, the factory isolates were indistinguishable from the human, clinical and veterinary strains. Further detailed metabolic profiling was undertaken using the biolog Microbial ID system. Multivariate analysis of the metabolic microarray revealed differences in metabolism of the factory isolate of *S.*Montevideo, based on its upregulated ability to utilise glucose and the sugar alcohol groups. The remainder of the serotype-matched isolates were metabolically indistinguishable. Temperature and humidity are known to influence bacterial survival and through environmental monitoring experimental parameters were defined. The results revealed *Salmonella* survival on stainless steel was affected by environmental temperatures that may be experienced in a food processing environment; with higher survival rates (D_{25}=35.4) at temperatures at 25°C and lower humidity levels of 15% RH, however a rapid decline in cell count (D_{10}=3.4) with lower temperatures of 10°C and higher humidity of 70% RH. Several resident factories strains survived in higher numbers on stainless steel (D_{25}=29.69) compared to serotype matched clinical and veterinary isolates (D_{25}=22.98). Factory isolates of *Salmonella* did not show an enhanced growth rate in comparison to serotype matched isolates grown in Luria broth, Nutrient broth and M9 minimal media indicating that as an independent factor, growth was unlikely to be a major factor driving *Salmonella* persistence. Using a live / dead stain coupled with fluorescence microscopy revealed that when no longer culturable, isolates of *S.*Schwarzengrund entered into a viable non-culturable state. The biofilm forming capacity of the panel was characterised and revealed that all were able to form biofilms. None of the factory isolates showed an enhanced capability to form biofilms in comparison to serotype-matched isolates. In disinfection studies, planktonic cells were more susceptible to disinfectants than cells in biofilm and all the disinfectants tested were successful in reducing bacterial load. Contact time was one of the most important factors for reducing bacterial populations in a biofilm. The genomes of eight strains were sequenced. At the nucleotide and amino acid level the food factory isolates were similar to those of isolates from other environments; no major genomic rearrangements were observed, supporting the conclusions of the phenotypic and metabolic analysis. In conclusion, having investigated a variety of morphological, biochemical and genomic factors, it is unlikely that the persistence of *Salmonella* in the food manufacturing environment is attributable to a single phenotypic, metabolic or genomic factor. Whilst a combination of microbiological factors may be involved it is also possible that strain persistence in the factory environment is a consequence of failure to apply established hygiene management principles.

Key words: *Salmonella*, Persistence, Food-Manufacturing, Survival, Temperature

You don't have to see the whole staircase just take the first step…

Success is a journey not a destination, the doing is more important than the outcome

For Mum and Dad

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1 Chapter 1- General Introduction

1.1 History of Salmonella

Salmonella was first discovered in 1885 by Daniel Elmer Salmon and Theobald Smith. Daniel Elmer Salmon was a veterinary surgeon who undertook research alongside his assistant Theobald Smith at the Bureau of Animal Industry, Washington D.C. USA. They found *Salmonella* in infected hogs believing it to be the cause of swine fever and initially named the organism ``Hog- cholera-bacillus''(Wray and Wray, 2000). Over the years after its discovery it was known by many other names too including TPE (typhoid parathyphus-enteritis) and *Erbethella typhi.* However in 1990, French scientist Joseph Leon Lignieres honoured Salmon by naming the entire swine group *Salmonella* (Brands *et al.*, 2009). *Salmonella* was discovered to be the cause of human typhoid fever and serum agglutination tests revealed that the bacillus agglutinated with serum from typhoid patients who were previously immunised with the typhoid bacillus (Wray and Wray, 2000).

1.2 Salmonella morphology and taxonomy

Salmonella are typically Gram-negative, rod-shaped bacilli that belong to the family Enterobacteriaceae (World Health Organization, 2013a). Other members of the Enterobacteriaceae family include *Escherichia*, *Shigella, Klebsiella* and *Citrobacter. Salmonella* are facultatively anaerobic, non-spore forming and possess peritrichous flagella for motility. The nomenclature of *Salmonella* is evolving and complex. The compound structure of the *Salmonella* bacterium and its characteristics allow the basis for the acknowledged naming scheme. Presently, the genus *Salmonella* is composed of two species: *S. enterica* and *S. bongori*. *S. enterica* is divided further into six subspecies: *enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI)* (Brenner *et al.*, 2000). Currently there are over 2600 different serotypes of *Salmonella* that have evolved from the initial `one serotype-one species' concept, proposed by Kauffmann and White on the basis of the serologic identification of three antigens; this concept relies on the antigenic variation in their lipopolysaccharide (LPS) O-antigen and flagella-associated H-antigens. The somatic (O) antigens are heat stable and resistant to alcohol, whereas flagella (H) antigens are heat-labile proteins. Serotypes Typhi, Paratyphi A, B and Dublin acquire a Vi antigen present on the surface envelope (Braoudaki, 2004). Serotypes possessing the Vi antigen are able to avoid phagocytosis as well as serum complement, causing an antibody response against the antigen

(Wain *et al.*, 2005). Surface antigens are also observed in other enteric bacteria such as *Escherichia coli, Shigella* and *Klebsiella* (Braoudaki, 2004).

Salmonella are one of the most important foodborne pathogens and are inevitably significant to the food industry (Manijeh *et al.*, 2008).They play a key role as pathogens in humans, being mesophilic and neutrophilic enables them to thrive in various moist environments. The primary habitat of *Salmonella* is the intestinal tract of humans and animals (both cold blooded and warm blooded) (Braoudaki, 2004). *Salmonella* species can establish predominantly in one particular host or they can be ubiquitous. For example Typhi and Paratyphi A are human serovars that can lead to severe diseases, usually related with the invasion of the blood stream. In these cases Salmonellosis occurs through human faecal contamination of food or water. whereas, ubiquitous *Salmonella* serovars like Typhimurium can cause a range of clinical symptoms- from an asymptomatic infection through mild diarrhoea to severe typhoid like syndromes in children and toxic infections in adults. Sources of *Salmonella* include factory surfaces, animal faeces, kitchen surfaces, raw poultry and seafood. They are also disseminated in the natural environment in water, soil and insects (Todar, 2012). It has been noted that *Salmonella* can proliferate in the natural environment (inside the digestive tract) and survive in water for numerous weeks and several years in soil, if conditions such as humidity, temperature, and pH are optimal.

1.3 Growth and survival characteristics

Salmonella spp. have fairly simple nutritional requirements and it has been noted that *Salmonella* can proliferate in the natural environment (inside the digestive tract) and survive in water for numerous weeks and several years in soil, if conditions such as humidity, temperature, and pH are optimal. Typically *Salmonella* are able to grow in broad temperature ranges from 5.5°C to 45.6°C with the optimal temperature being 35–43°C (ICMSF, 1996). However, growth in some strains has been observed at temperatures as low as 3.5°C (Morey and Singh, 2012). Furthermore it has been demonstrated that *Salmonella* can survive for long periods of time on frozen foods. Strawn & Danyluk (2010), conducted a study investigating the survival of *E.coli* and *Salmonella* on mangoes and papayas. Results revealed that *Salmonella* was able to survive on mangoes stored at 4°C for 28 days; interestingly survival on frozen mangoes and papayas stored at -20°C was 180 days (Strawn and Danyluk, 2010).

Being a neutrophile, *Salmonella* spp. is capable of growing in pH ranging from 3.8–9.5, with an optimum growth pH being between 7 and 7.5 (ICMSF, 1996). The lowest pH *Salmonella* are able to grow is dictated by factors such as; the temperature, availability of salt and nitrate and the presence of specific acid. Volatile fatty acids are more bactericidal compared to organic acids like citric, acetic and lactic acid. If the pH is not within range there is a possibility of cells becoming inactivated, however this will not occur instantaneously as it has been specified that cells can survive for elongated periods in acidic products (Bell and Kyriakides, 2002; Jay *et al.*, 2003). Importantly, the optimum water activity *(Aw)* for *Salmonella* growth is 0.99 with the lower limit at 0.93. This allows *Salmonella* to survive years in foods with a low *Aw* like chocolate, pepper and peanut butter (ICMSF, 1996; Podolak *et al.*, 2010).

Salmonella are facultative anaerobes therefore can grow in the absence of oxygen (Jay *et al.*, 2003). Furthermore *Salmonella* are highly susceptible to preservatives used in foods such as benzoic acid, sorbic acid and propionic acid, this susceptibility is further enhanced when combined with factors such as lower temperatures and pH (ICMSF, 1996; Banerjee and Sarkar, 2004). The ability of *Salmonella* to survive in harsh, nutrient limited conditions, from dry external environments to the acidic conditions of the stomach, is key in enabling them to persist in the conditions of the lower intestinal tract and the embedded proteins in the outer membrane play an essential role in the transportation of nutrients when required (Wray and Wray, 2000).

1.4 Pathogenesis and virulence

Pathogenicity and virulence are important terms when understanding how microorganisms overcome barriers to cause disease. The term pathogenesis refers to the mechanism that causes a disease; it also describes the origin and development of disease, whereas, virulence is derived from the Latin word `virulentus' which translates to `poisoned wound' and is defined as the degree of the pathogenesis (Mistry, 2012). *Salmonella* are able to thrive in humans due to their complex pathogenicity and their interaction with host cells. They possess a series of phenotypic and genotypic virulence factors that enable them to overcome host defences. Following ingestion of contaminated foods or water, *Salmonella* must survive the low pH as well as changes in temperature and oxygen levels in the stomach and colonise the small intestine. *Salmonella* then multiply, invade and adhere to the host intestinal epithelial mucosa. Following the penetration of the epithelial cell barrier the bacteria are able to spread through

the lymphatic system and the blood stream to reach the liver and spleen (Clements *et al.*, 2001).

1.4.1 Salmonella Pathogenicity Island encoded type III secretion system

Gram-negative bacterial pathogens are able to use type III secretion systems to provide virulence factors to the host cells and interfere with host cell signalling pathways. *Salmonella* host interaction is a complex process that involves a range of genes for virulence, which are located on 'pathogenicity islands' in the chromosome and believed to have been acquired via horizontal transfer from other organisms. At present, the virulence of five *Salmonella* Pathogenicity Islands (SPIs) has been described in detail; SPI1, SPI2, SPI3, SPI4 and SPI5 (Marcus *et al.,* 2000). *Salmonella* are intracellular pathogens so are able to hide within the host cells and spread from cell to cell. *Salmonella* have mechanisms to avoid the host cell's intracellular defence mechanism and multiply to infect other cells (Liu *et al.*, 2012). A range of effector proteins are involved in the invasion of the intestinal epithelial cells that regulate an inflammatory response and initiate symptoms such as diarrhoea (Tang *et al.*, 2001; Tang *et al.*, 2014).

1.4.2 Infectious dose

Generally a dose of 10^3-10^7 cells can cause Salmonellosis and potentially lead to toxic infections. However in cases such as when *Salmonella* is present in food, a host can be infected with as few as 10^2 cells, as the food acts as a protective layer, shielding *Salmonella* from the severe acidic conditions of the stomach. The low pH in the stomach effectively eliminates approximately 99% of *Salmonella* cells, the remaining 1% pass into the small intestine and are exposed to further environmental change.

1.5 Salmonellosis clinical features

Salmonella infections in humans cause a range of symptoms and usually depend on the nature of the contamination, the infectious dose and health of the host. The clinical symptoms of Salmonellosis occur within 12-72 hours after infection and as figure 1 shows include: diarrhoea, abdominal cramps, vomiting and fever. Gastroenteritis is a localized infection and usually lasts 4-7 days, in most cases it is self-limiting that clears without medication, although rehydration is an important factor when diarrhoea is severe. The HPA (2011) reports that in the UK a majority of cases are caused by non-typhoidal strains *S.* Typhimurium and *S.* Enteritidis.

Figure 1 An illustration of how *Salmonella* enters and colonises the body

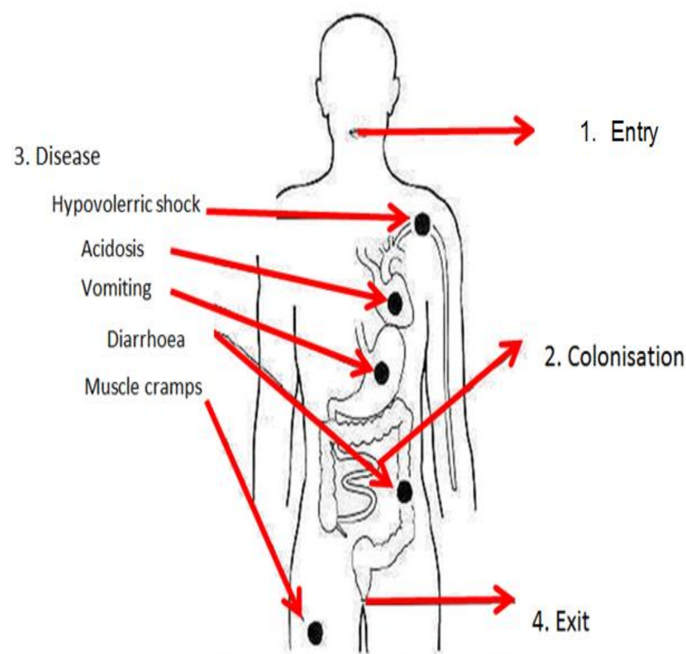


Figure 1: an illustration of the route by which *Salmonella* enters and colonises the body, with a list of some of the possible symptoms of infection adapted from Giannella (1996).

1.6 Antibiotic treatment of Salmonellosis

Although most cases of *Salmonella* are self-limiting, in the case of severe infection caused by *S.*Typhi and Paratyphi, which lead to high fever, rapid treatment is required. For adults the antimicrobials that are widely used for treatment are the group of fluoroquinolones; since they are reasonably inexpensive, are absorbed well orally and compared to former drugs are more rapidly and reliably effective. For children that present with serious infections, third-generation cephalosporins are injected. Alternatively depending on severity of infection, earlier antibiotics are administered such including ampicillin and gentamicin. However there is increasing evidence of resistance to earlier antibiotics (World Health Organization, 2013b; Le Hello *et al.*, 2013).

1.7 Epidemiology

1.7.1 Burden of Salmonella worldwide

Surveillance data rely on serotyping as a tool to detect outbreaks, monitor trends and to characterise potential foods and animal as reservoirs of human infections (Herikstad *et al.*, 2002). The global incidence of *Salmonella* is estimated at 93.8 million cases annually, of which a staggering 80.3 million are thought to be associated with foodborne illness. However, the available surveillance data are limited to reporting trends of foodborne pathogens in developed countries (Majowicz *et al.*, 2010; Newell *et al.*, 2010). For example, a study conducted in the US in 2000, estimated 1.4 million non-typhoidal *Salmonella* infections of which only 168,000 individuals visited a GP, 16,430 were hospitalized and 582 died (Mead *et al.*, 1999; Herikstad *et al.*, 2002). Similarly a study conducted in the UK by Adak *et al.* (2002) showed 41, 616 cases of non-typhoidal *Salmonellas* occurred each year, 15,036 laboratory confirmed cases were reported, as a result of which 1,516 individuals were hospitalized and 119 died (Adak *et al.*, 2002; Herikstad *et al.*, 2002). When taking into consideration population sizes of the US (293 million) and the UK (60 million) the ratio of deaths in the UK is actually high.

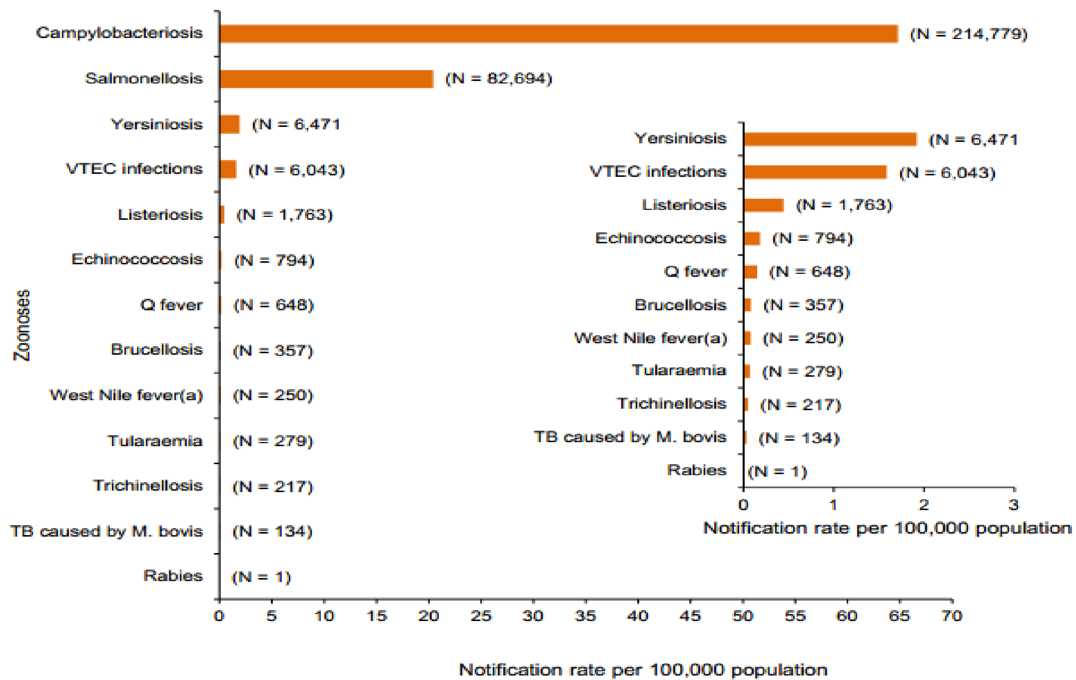
One study looked at data from various developing countries and expressed that the true burden of *Salmonella* infection is not highlighted, as data collected was provisional since for some regions cases were not even documented or were unavailable to the public. This included those countries with a large proportion of the global population, such as Asia and South America, where water supplies are not in abundance and sanitation is generally poor. In these regions, it is likely that foodborne infections are lower in comparison to the proportion of cases reported due to contaminated water. Globally, mass production and circulation of foods allows the rapid dissemination of pathogens, thus improving food safety and the implementation of food safety interventions is required at a global level not just a European level (Majowicz *et al.*, 2010).

Overall worldwide, reports have suggested a decrease in *Salmonella* infections over the years. This is likely to be attributed to better sanitation, access to clean water, improved living conditions and vaccination. However outbreaks of *Salmonella* linked to undercooked meat and cross contamination are still of significance, as after *Campylobacter* it remains the second most common cause of food poisoning (FSA, 2008; Little *et al.*, 2008).

1.7.2 Incidence of Salmonella in Europe

In Europe, traditionally trends show the incidence of *Campylobacter* has been much higher than that of *Salmonella* (Herikstad *et al.*, 2002). As Figure 2 from the EFSA report highlights, the number of cases of *Campylobacter* continued to remain high with 214,779 cases reported in 2013; this is an increase of 511 cases from the report in 2012.In comparison the cases of human *Salmonellosis* revealed a 7.9 % decrease in the EU notification rate compared with 2012, this is a statistically significant decreasing trend in the European Union observed between 2009-2013. The highest number of cases were reported during the summer months and in total, 82,694 confirmed human cases were reported in 2013. The reports also revealed that some serovars were more prevalent in certain foods and animals, therefore identifying the serovar linked to the outbreak is valuable in identifying the source of the outbreak (EFSA and ECDC, 2012; EFSA and ECDC, 2015).

Figure 2 Reported notification rates of zoonosis in confirmed human cases in the EU, 2013



1.7.3 Surveillance of foodborne infections in the UK

The Public Health Act 1984 states that *Salmonella* outbreaks in the UK must be reported to the Consultant in Communicable Disease Control (CCDC). They will then assess the available information from individuals and refer to them as `cases' and search for any associations between cases of infection; for example, similarities in symptoms, and consumption of specific food products or food places. The data are then anonymised and forwarded to the Office of Public Censuses and Surveys (OPCS) to generate national food poisoning statistics, which are then published in the Communicable Disease Report by the PHE (formally known as HPA) and the Communicable Disease Surveillance Centre (CDSC). For further identification and confirmation, isolates are also sent to the Laboratory of Enteric Pathogens (LEP), which is the national reference laboratory. Once analysis is complete, results are relayed back to the CDSC and contribute to the weekly-published figures. (Hilton, 1997; (Mistry, 2012) However, as the Intestinal Infectious Disease (IID) reports reveal, the available data fail to show a true representation of the burden of *Salmonella* , as it is a self-limiting infection, many individuals do not seek medical advice and self-diagnose, consequently these cases are not included in epidemiology reports. Overall reports have suggested a decrease in *Salmonella* infections over the years. This is likely to be attributed to better sanitation, access to clean water, improved living conditions and vaccination. However outbreaks of *Salmonella* linked to undercooked meat and cross contamination are still of

significance as after *Campylobacter* it remains the second most common cause of food poisoning (FSA, 2008; Little *et al.*, 2008).

Figure 3 Notification pyramid for gastrointestinal infections

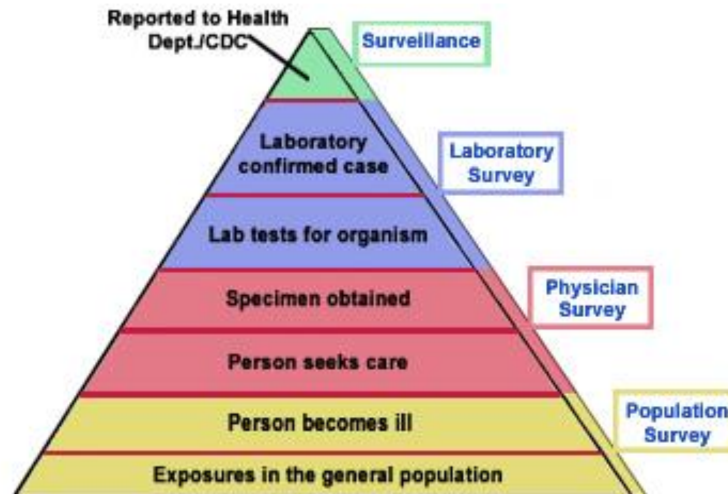


Figure 3: an illustration of a notification pyramid for gastrointestinal infections. It shows that the number of cases that surveillance programmes are made aware of is only a small sub section of the actual number individuals affected. As only a small proportion of affected individuals seek medical attention, the level of specimens collected and sent to the laboratory to confirm the diagnosis is even lower which consequently reflects on the figure of confirmed cases to health departments.

1.7.4 The Infectious Intestinal Disease (IID) survey

Obtaining true figures representing the burden of disease is important. The first Infectious Intestinal Disease (IID) Study conducted in England (1993-1996) (Wheeler *et al.*, 1999), highlighted the burden caused by gastrointestinal infections and provided models to establish the actual number of infections in the community in comparison to reported figures. Surveillance of foodborne infection is based on data collected for five key pathogens; *Salmonella, Campylobacter, Clostridium perfringens*, *Escherichia coli* O157 and *Listeria monocytogenes.* A more recent IID study (2008-2009) (Tam *et al.*, 2012), provided a more accurate reflection of the relationship between disease burden in the community and official published statistics.

Figure 4 Specific estimates of proportion foodborne from reported outbreaks, UK 2001-2008

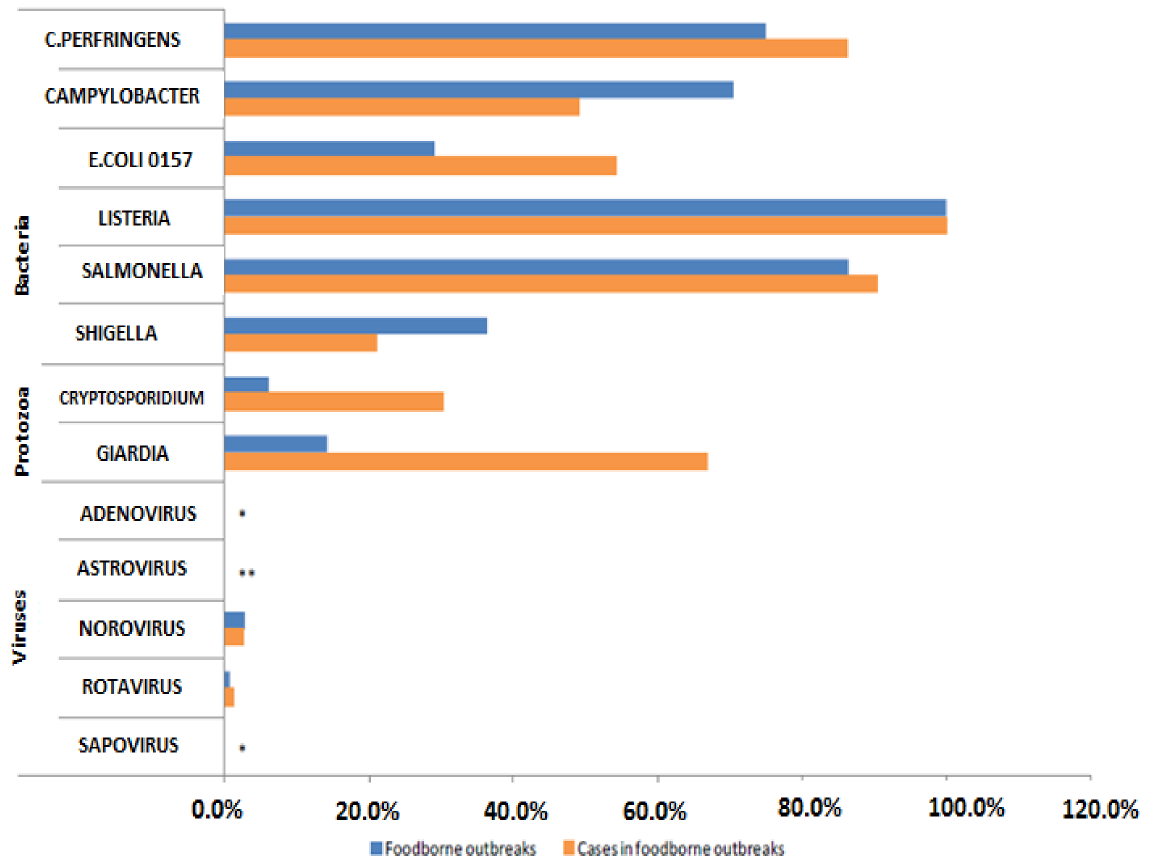


Figure 4 shows estimates of the percentage of gastrointestinal outbreaks reported in the UK between 2001 and 2008. The blue bars indicate those associated with foodborne transmission the orange bars represent the percentage of cases within reported outbreaks that were associated with foodborne outbreaks. The figures indicate that for *Listeria,* all reported outbreaks were associated with foodborne transmission. For *Salmonella* 90% of total reported cases were associated with foodborne outbreaks. Similarly, 86% of total cases of *C.perfringens* were associated with foodborne outbreaks whereas for *Campylobacter* only 49% of reported cases were foodborne (Tam *et al.*, 2012). These data illustrate the importance of *Salmonella* as a foodborne vehicle. Figure 5 focusses on the data obtained for cases of *Salmonella* in the UK, and draws comparison between figures reported from the IID1 and IID2 study.

Figure 5 Reporting Pattern of IID due to *Salmonella* in England (IID1 & IID2)

Figure 5: the information in blue represents data collected about *Salmonella* incidence from the first infectious intestinal disease (IID1) study conducted in 1993-1996, whereas the information presented in red shows data from the second infectious intestinal disease (IID2) study conducted more recently in 2008-2009. Incidence of *Salmonella* IID appears to have decreased dramatically since the first study was conducted however it still equates to 17 million cases annually. Incidence in the community was 43% higher in IID2 than IID2; however of the total cases reported only 2% sought medical advice from GP's which is eight times lower than figures documented in IID1. Consequently, these figures were reflected in the more than 4-fold decrease in the frequency of reports to national surveillance for *Salmonellosis* (Wheeler *et al.*, 1999; Tam *et al.*, 2012).

1.7.5 Salmonella cases in the UK by serotype

Recently, the European Food Safety Authority (EFSA), and the European Centre for Disease Prevention and Control analysed foodborne infection data from 27 EU countries; findings highlighted that 9670 confirmed cases of *Salmonella* were reported in the UK in 2010 (EFSA and ECDC, 2012). This is a considerable decline from previous years; in 2009 10,479 cases were reported and 14,144 cases in 2006

Figure 6 Human isolates of *Salmonella* reported for Infections in England and Wales, 2000 – 2010

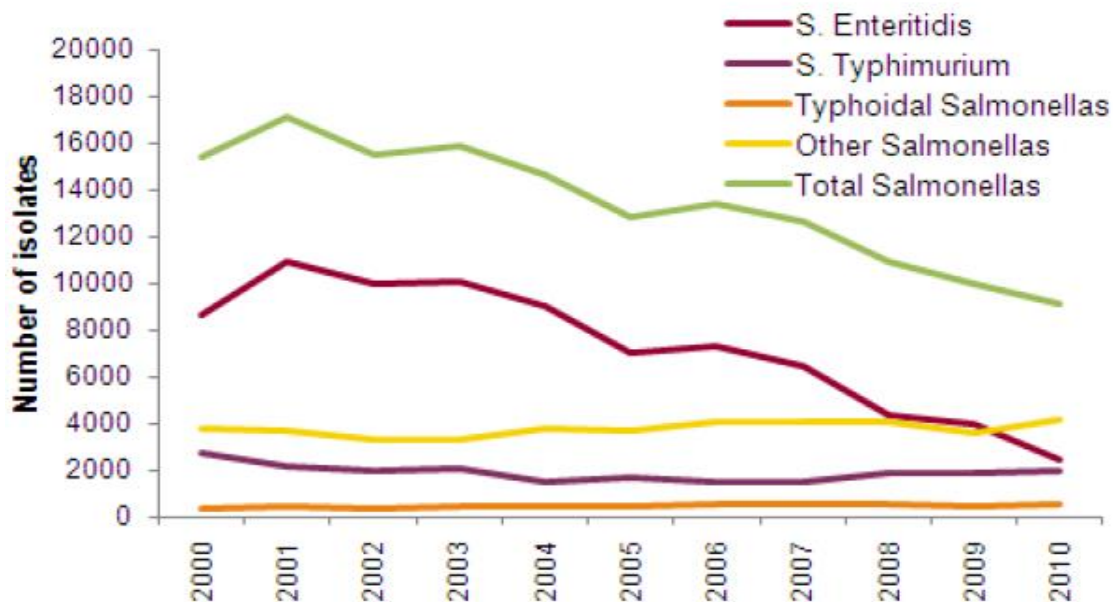


Figure 6 shows the incidence of Salmonellosis in humans by serotype and shows the trend since the year 2000 (HPA, 2011). It reveals a noticeable decrease in cases of both *S.*Typhimurium and *S.*Enteritidis, however cases of Typhoidal *Salmonella* increased from 331 to 569 and cases of `other' serotypes increased from 3798 to 4161 in 2010. This may be attributed to increased international travel over the past decade. Reports from the food industry have also indicated an increase in the presence of rare serotypes. Inevitably with improvements in sanitation facilities, education and awareness, the overall incidence in developed countries has declined substantially.

1.7.6 Incidence of Salmonella cases in the UK by region

Figure 7 Human isolates of *Salmonella* reported for Infections in England and Wales, 2000 – 2010

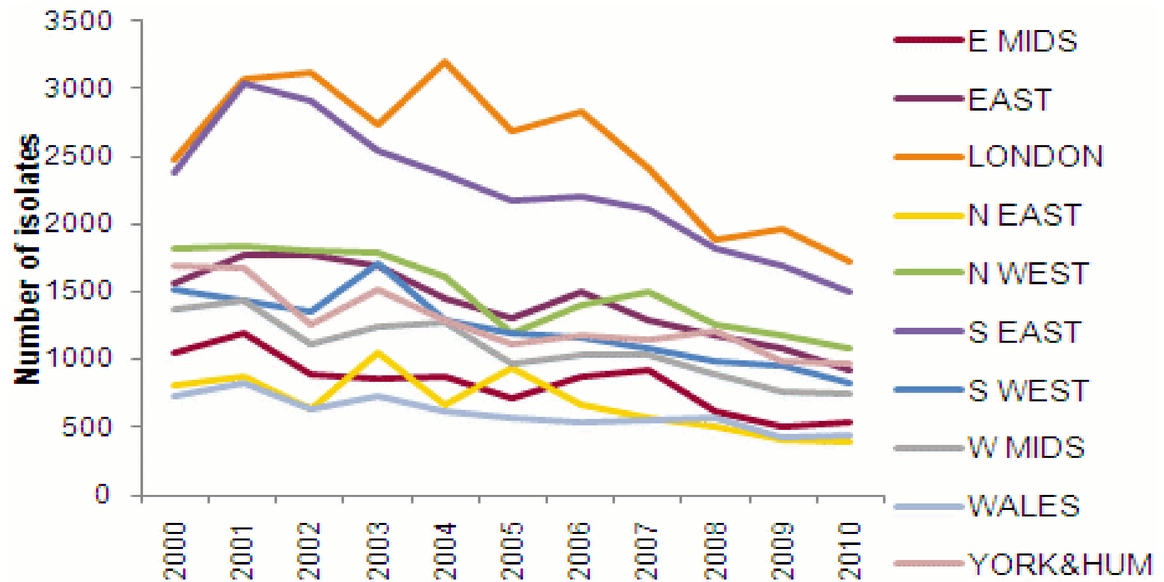


Figure 7 shows human isolates reported to the Health Protection Agency Centre for infections per 100,000 populations by region in England and Wales in this time period (HPA, 2011). Since 2000, London has had the highest incidence of *Salmonella;* in 2010, 1721 cases were reported compared to only 390 in the North east of England. This could be attributed to 0London being urbanised; also it has a diverse population and a range of people from different cultures, and a high exposure of foods from different countries.

The EFSA report showed that in August to September there is usually a peak in the number of reported *Salmonella* cases and a rapid fall in winter months. This pattern was noticeable for all age groups, which supports the impact of outdoor temperature on proliferation of bacteria in foods and environment (EFSA and ECDC, 2012).

1.7.7 The changing epidemiology of Salmonella

The incidence of *Salmonella* was lowest during World War II in the 1940s, due to rationing and poor access to meat and eggs. However, 1953-1954 saw a rapid increase in animal slaughter, a rise in meat and egg consumption and consequently a hike in cases of salmonellosis. Farming patterns changed drastically with an initial demand of pigs and poultry followed by beef calves, veterinians warned that new farming methods played a pivotal role in the increased spread of *Salmonella* infection. However outbreaks of *Salmonella* were not solely linked to animals; 1959 also highlighted a phenomenal surge in *Salmonella* infections linked to hens' egg*s.* As the number of outbreaks increased, the serotype *S.*Enteritidis emerged as a dominant causative agent of food poisoning worldwide (Hardy, 2004). This was attributed to the ability of *S.*Enteritidis to infect poultry asymptomatically, particularly in laying hens, which made the detection of disease difficult. Early reports indicate *S.*Enteritidis may have existed in the rodent population linked to hen's houses during the 1930s.

During the 1980s *S.*Enteritidis phage type 4 (PT4) was identified as the most prevalent serotype and the leading cause of foodborne infection was linked to egg shells. A majority of these cases were due to the consumption of eggs which had become contaminated due to bacteria on the shell; most likely by penetrating through the eggshell or the contamination of eggs during breaking. Infection of eggs and chicks may occur through vertical transmission from the spleen of the hen. The infection can spread down the reproductive tract as the number of cells increase at sexual maturity; the ovaries may become infected if strains spread from the cloacae to the reproductive organs, playing an important role in re-infection (Shivaprasad, 2000). As a preventative measure, all eggs in the UK and USA were pasteurised and washed to effectively remove faecal contamination prior to packing (Cogan and Humphrey, 2003; Hardy, 2004).

The initial decline in cases of *Salmonella in* the 1990s, was impacted by a statement made by former minister Edwina Curry, who through her comments in an ITN interview highlighted that "most of the egg production in this country sadly is now infected with *Salmonella*". Although this was an exaggeration, it led to a drop in consumer confidence levels and sales of eggs plummeted 60% overnight (BBC, 1988). In turn this lowered the risk of cases caused by *S.* Enteritidis PT4, however loss of income compelled farmers to slaughter four million hens and destroy 400 million unwanted eggs (Intervet, 2014). Over time egg sales improved and consumer confidence was restored, thus increasing the rate of infection caused by *Salmonella.*

Over time, intervention strategies have been implemented, including education, environmental monitoring disinfection, and most importantly vaccination (Mistry, 2012). A second decline in rates of infection occurred with the introduction of the vaccine Nobilis Salenvac, which was administered to laying chickens. This vaccine was effective in reducing human incidence of *Salmonella* Enteritidis by 63% (Woodward *et al.*, 2002). Studies have proved Salenvac transfers passive protection between the breeder hen, her eggs and chicks, which are vital in protecting eggs from *Salmonella.* Salenvac is an inactivated vaccine that is injected in the hen and stimulates the production of maternally derived antibodies against *S.*Enteritidis in the chick before it hatches (Intervet, 2002; Woodward *et al.*, 2002) As a result, in the UK all eggs laid from vaccinated hens are labeled with a red lion stamp and a best before date. This lion code provides registration and traceability for the whole production chain and allows audits to be conducted for each step , it has also prompted the enforcement stricter hygiene, time and temperature controls (Intervet, 2014).

However this vaccine was not a sustainable solution as the import of eggs from other counties cannot be controlled and new serotypes of *Salmonella* are continually emerging, furthermore, multi resistant strains of *S.* Typhimurium DT 104 have also been reported (Intervet, 2002). In 2009, EU legislation stated that eggs would not be used for direct human consumption as table eggs unless they originated from a commercial flock of laying hens subject to a national *Salmonella* control programme (EFSA and ECDC, 2012).

However, in 2011, The Food Standards Agency (FSA, 2011) published a report detailing an outbreak of Salmonellosis in the UK that was linked to eggs from a farm in Spain, these eggs were largely supplied to catering companies. The HPA confirmed that the outbreak was caused by *S.*Enteritidis (PT) 14b and overall it affected 262 people (FSA, 2011). Recently, Nobilis extended their research and produced Salenvac T, this broadens the protection range by protecting against *Salmonella Enteritidis* PT4 and *Salmonella* Typhimurium DT104. Contrarily to *Salmonella* grown on agar plates, in chickens nutrients such as iron are less readily available, to acquire iron *Salmonella* express Iron Regulated Proteins (IRPs) on their surface, which are then recognised by the chicken's immune system as antigens. Therefore to mimic these conditions, Salenvac T vaccine is manufactured with a shortage of iron, and allows the vaccinated chicken to generate additional antibodies against these IRP*S.* The IRP antibodies then provide greater protection against natural challenge (Intervet, 2002; Intervet, 2004).

1.8 Routes and reservoirs of transmission

Salmonella can infect a range of hosts other than humans including animals and insects. It is a key concern for meat production sites as poultry is recognised as a global *Salmonella* reservoir and contamination can occur at any stage of the production chain, posing as a major route of transmission to humans. Extensive research has been conducted exploring the control of pathogen transfer at each stage of the manufacturing process in food environments, in addition to strategies which have been implemented to reduce the risk of cross contamination such as effective monitoring, disinfection and vaccination programmes (Heyndrickx *et al.*, 2002; Van Immerseel *et al.*, 2009). *Salmonella* infection can occur through a range of routes including contaminated foods, water, a range of contact surfaces and the environment.

Figure 8 An illustration showing the array of possible routes of *Salmonella* transmission between reservoirs

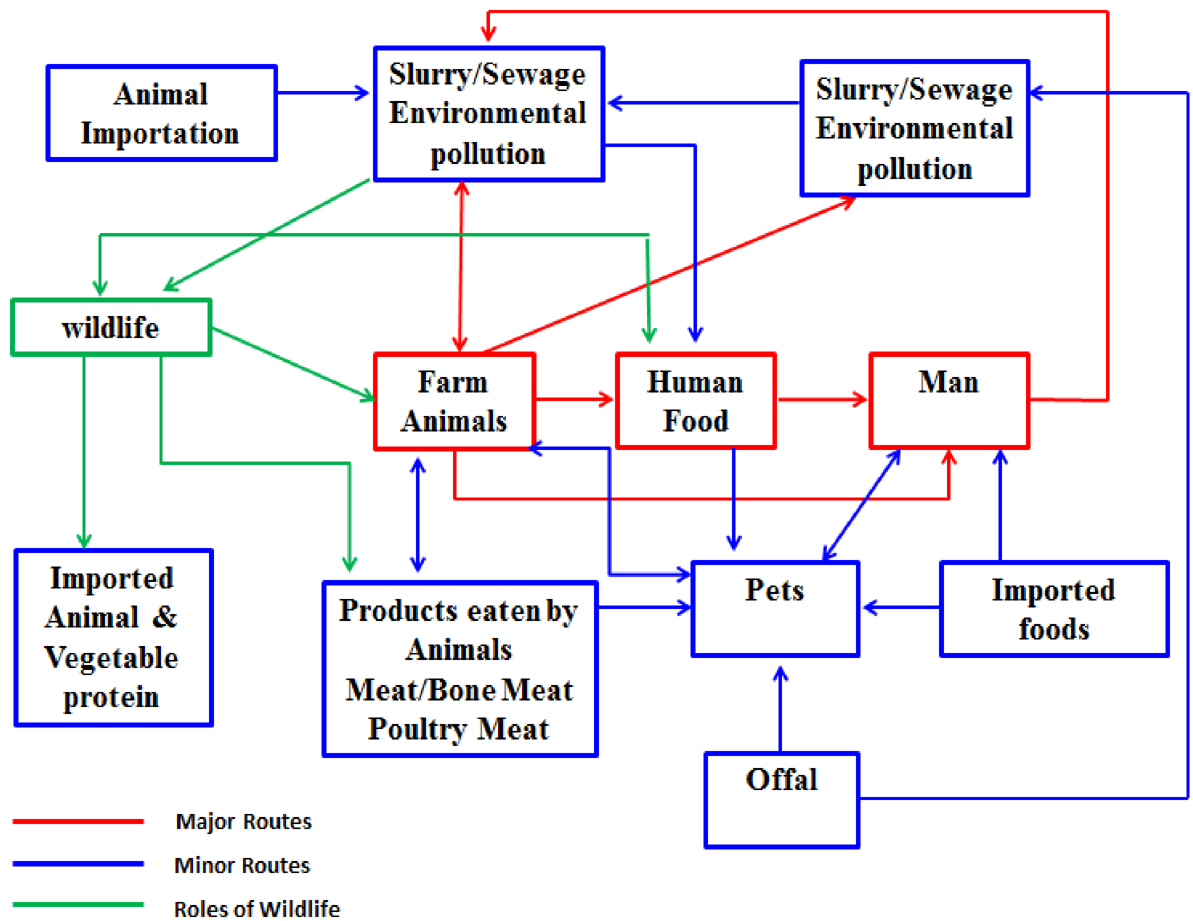


Figure 8 summarises the possible routes of *Salmonella* transmission (adapted from Hilton, 1997). The red lines indicate major routes of transmission, blue lines indicate minor routes and green indicates the role of wildlife. The transmission route of *Salmonella* infection is complex and challenging to control as there are many areas of exposure, including the import of foods, interaction with animals and pets (Le Hello *et al.*, 2013). One of the major routes by which *Salmonella* is spread is through contaminated food, frequently meat and dairy products from farm animals (Jayarao *et al.*, 2006). During the butchering process, raw beef and poultry may become contaminated through faecal contact with food. Foods can also be contaminated through infected food handlers who do not wash their hands adequately after visiting the toilet. A possible route for fish to become contaminated is through water which has *Salmonella* present. The pathogenic cells harbour in food that is not washed before consumption or food that is undercooked.

1.9 Salmonella in food products

As a ubiquitous organism, *Salmonella* may occur naturally in the environment and can pass via many routes in the food chain from producers to consumers (Gormley *et al.*, 2011). *Salmonella* contamination has been implicated in a range of food products including poultry, fish eggs, dairy, vegetables and dry foods. In the EU, one of the highest categories of isolation is from fresh broiler meat and turkey meat with an average level of 5.5% and 8.7%, in fresh pig and bovine meat, the figures revealed 0.7 % and 0.2 %, respectively. In poultry, *Salmonella* causes a high level of sub clinical infections and can spread between herd and flock without being detected; animals can become silent carriers of the organism. The association of *Salmonella* in poultry populations is considered as the main risk factor for presence of *Salmonella* in table eggs and poultry meat (Giaouris *et al.*, 2012; Hugas and Beloeil, 2014). Although almost 25 years of national guidance on the safe handling and use of eggs has been available, eggs still pose as a vehicle of transmission for *Salmonella* infections in food service establishments, implying government guidance is not being precisely followed (Gormley *et al.*, 2011). Table 1 lists foodborne outbreaks recorded in the UK, and the causative agent by implicated food vehicle.

Table 1 Foodborne outbreaks recorded in England and Wales from 1992 to 2008 showing causative agent by implicated food vehicles

Table 1: lists some food products that were implicated with foodborne infection 1992-2008. Of the total 1836 foodborne cases reported, 928 were attributed to *Salmonella.* 54.2% of poultry meat-linked outbreaks, 89.9% of dessert consumption outbreaks and 58.6% of egg associated outbreaks were also accounted for by *Salmonella.* The figures above show that almost 20% of the 928 cases of *Salmonella* were attributed to miscellaneous foods highlighting the risk surrounding buffets, where cross contamination may occur due to food sitting outside for long periods, people touching foods and utensils with unwashed hands and poor temperature maintenance (Gormley *et al.,* 2011)

1.9.1 Processing of raw ingredients

The raw ingredients entering food manufacturing sites come from a range of sources and suppliers. They may include relatively unprocessed ingredients like meat, milk and eggs. These ingredients are likely to be carrying organisms, and the food environment itself may have organisms present, these are likely to be moved around with personnel and resources in the environment. It would be impractical, and highly impossible for food processing factory environments to be sterile. In most cases the raw ingredients undergo various levels of processing before the end consumer products are produced, packaged and stored in a manner to prevent contamination. If the processing is not done on site then manufacturers rely on the risk assessment documentation provided by the supplier which indicates that the presence of food-borne pathogens is of low risk. Monitoring the status of incoming products is vital as cases of contaminated end products have been as a result of a poor selection and monitoring of raw ingredients (Finn *et al.*, 2013a). The guidelines for assessing the microbiological safety of ready to eat foods, states that *Salmonella* must be absent in 25g of food (HPA, 2009).

1.9.2 Ready to eat foods

Ready-to-eat (RTE) foods may act as vehicles for foodborne disease; they are classified as products that do not require any additional processing to being consumed (Carrasco *et al.*, 2012). For these foods, there are no further steps involved to remove any potential pathogenic microorganisms which may be present. Chocolate, peanut butter and some meat products fall into the RTE low moisture category. For products that require cooking prior to consumption, the instructions on the packaging must clearly state this with extra consideration for heat tolerance of pathogens that are able to proliferate in low moisture foods compared to those in high moisture foods. Although measures have been implemented to stop the transfer of pathogens, there is no assurance that consumers will comply with instructions, therefore the manufacturer should implement strategies for the elimination of pathogens in foods distribution. In the case of products which may be eaten raw, the manufacturer has a responsibility to ensure good quality ingredients are utilised and packaging is clearly labelled to lower the risk of potential contamination (Finn *et al.*, 2013a). Studies have revealed factors such as utensils, food handlers, aprons and work surfaces all pose a high risk of contamination of RTE foods (Christison *et al.*, 2007). An important risk factor is the use of blades during cutting of meats, as each slice has the possibility of disseminating high numbers of microbes (Pérez-Rodríguez *et al.*, 2007).

1.9.3 Low (A_w) Foods

For many years food products have been preserved via drying, low moisture foods have an increased shelf life and are considered stable for years. Products in this category exhibit a reduced water activity (A_w); this is expressed as the ratio of the vapour pressure of water in a food matrix versus that of pure water at the same temperature and is affected by factors such as temperature at storage and composition (Finn *et al.*, 2013a). There are a range of products which fall into the natural low (A_w) category including cereals, nuts and honey. Whereas chocolate, powdered infant milk, pasta and peanut butter are high moisture foods which have undergone a drying process. Despite the common misconception regarding contamination of low water activity foods, these products are subject to microbial contamination and growth of organisms such as *Salmonella* may proliferate, posing a risk to consumers and the suppliers in the food chain (Carrasco *et al.*, 2012; Finn *et al.*, 2013a).

1.9.4 Salmonella outbreaks linked to direct and indirect contact with low moisture foods

Salmonella association with a variety of different foods means that there is a risk of exposure either through the direct consumption of contaminated foods or indirectly by the consumption of food prepared in a contaminated environment like a kitchen or even handling contaminated pet food and not washing hands adequately. Table 2 highlights some of the cases of *Salmonella* infection caused by either the direct consumption or indirect contact with contaminated low-moisture foods. The details of some of these outbreaks are detailed later in the text.

Year	***Salmonella* serotype(s)**	**Product**	**Location**	**Number of people affected**
1973	Derby	Powdered milk	Trinidad	3000
1974	Eastbourne	Chocolate	Canada	95
1982	Napoli	Chocolate	UK	245
1985	Ealing	Powdered infant formula	UK	76
1987	Typhimurium	Chocolate	Norway, Finland	361
1993	Rubislaw, Saintpaul, and Javiana	Potato chips	Germany	1000
1995	Senftenberg	Infant food	UK	5
1996	Enteritidis PT4	Marshmallow	UK	45
1996	Mbandaka	Peanut butter	Australia	15
1998	Agona	Cereal	USA	209
2000	Enteritidis PT30	Almonds	USA, Canada	168
2001	Stanley and Newport	Peanuts	Australia, Canada, UK	109
2003	Agona	Tea	Germany	42
2005	Agona	Powdered infant formula	France	141
2006	Tennessee	Peanut butter	USA	628
2008	Agona	Cereal	USA	28
2008	Typhimurium	Peanut butter	USA, Canada	714
2009	Montevideo	Red and black pepper	USA	272
2011	Enteritidis	Turkish pine nuts	USA	43
2012	Infantis	Dry dog food	USA	49
2012	Bredeney	Peanut butter	USA	42
2013	Montevideo/Mbandaka	Tahini pasta	USA	16

Table 2 A list of outbreaks of *Salmonella* infection after consumption or contact of low-moisture foods (Finn *et al.*, 2013a)

1.9.5 Outbreaks of S.Senftenberg, S.Anatum, and S.Kedougou in infant food

Numerous outbreaks of *Salmonella* linked to contaminated infant food have been reported worldwide. In 1995, an outbreak of *S.*Senftenberg associated with infant cereal was identified in the UK. Following investigation, the manufacturer revealed that *S.*Senftenberg was isolated from a cereal batch in the previous year but the batch was not distributed. In light of the outbreak the company put preventative measures in place to reduce any repeat occurrence (Rushdy *et al.*, 1998).

However, in 1997, The Public Health Laboratory Service (PHLS, 1997) recognised an increase in the number of *S.*Anatum cases in the UK and conducted an investigation into the source of the outbreaks, including data from European counties. As a high proportion of patients were infant's, it indicated that baby food may be the vehicle of transmission. Results showed that over the four months, in England 17 cases were reported and of those 15 had been fed the implicated infant milk formula. Food consumption histories were recorded and molecular analysis was performed on the *S.*Anatum isolates using pulsed-field gel electrophoresis. Once the data were collated, there was strong evidence to show a particular formula of milk, manufactured in France, was the source of the *S.*Anatum outbreak. It resulted in the factory closing down for cleaning and the infant formula milk produced from the contaminated batch was withdrawn from distribution. The *Salmonella* scare was particularly worrying as it involved children and changed the public's view of the product (PHLS, 1997).

Furthermore, recent reports from Spain revealed an increase in isolated cases of *S.*Kedougou; this is a relatively rare serotype as between 2002-2007 the National Centre of Microbiology (NCM) recorded the mean number of *S.*Kedougou strains isolated from humans were just three per year. Investigations of the 42 confirmed cases revealed 31 were infants under the age of one, and all had consumed the same brand infant formula milk. As a result, food safety authorities recalled five batches of the milk on 26 August 2008 after which no further cases emerged**.** Previous literature reveals only two other cases linked to *S.*Kedougou, the first in the UK in 1992 associated with cooked meat and the second case was observed in 2006 in Norway and was associated with salami (Rodriguez-Urrego *et al.*, 2010).

1.9.6 Outbreak of S.Typhimurium and S.Montevideo associated with chocolate

In 1987 an outbreak of *S.* Typhimurium associated with contaminated chocolate produced by a Norwegian company, resulted in 349 cases of infection. A majority of the infected were young children who developed acute haemorrhagic diarrhoea. Surprisingly laboratory findings showed less than or equal to 10 cells per 100 g of chocolate in about 90% of the positive samples obtained from retail outlets, suggesting that such a low dose is sufficient to cause symptomatic disease (Kapperud *et al.*, 1990).

In 2006, an outbreak of *S.*Montevideo in the Cadburys plant poisoned 37 people in the UK. After interviewing 15 of the 37 people affected, HPA found that 13 people consumed Cadburys products thus linking the outbreak to Cadbury's. Following the announcement by the Food Standards Agency, Cadbury's recalled one million chocolate bars from the UK market. The HPA conducted an investigation in the plant and found that samples contained the same *S.*Montevideo strain. The outbreak cost the company £5 million to withdraw the seven products and £20 million in loss of sales due to lack of customer confidence (ElAmin, 2006).

Recently an outbreak of *S.*Typhimurium was associated with King Nut peanut butter; it affected 714 people (CDC, 2009). This caused wide spread unrest as King Nut is produced by Peanut Corporation of America and the King Nut peanut butter was not directly sold to people but it was distributed to food manufacturers across America and in many other countries. Peanut butter and peanut paste is a key ingredient in a range of food products including: cookies, crackers, cereal, ice cream, pet foods and many other foods. This led to severe implications for the company when they had to recall the product as over 2833 peanut-containing products had to be tracked from various countries. Laboratory investigations showed that in a majority of cases the contamination was associated with the container of the peanut butter (CDC, 2009)

1.9.7 Contamination of S.Livingstone implicated with fish factory

Between 200-2001, Norway and Sweden observed an outbreak of gastrointestinal infection caused by *S.*Livingstone affecting 60 individuals; of which three died and 22 were hospitalised. Laboratories analysed faeces, urine and blood samples and further investigation via pulsed field gel electrophoresis (PFGE) saw the outbreak strain was identical to a previously isolated strain from sludge sewage earlier that year. The contamination was sourced back to a fish factory and the ranges of fish products were recalled and officials issued a public health warning. In 1991, *S.* Livingstone infection was documented in Scotland

however the source was not identified, a few years later in 1996, a large outbreak was observed in Europe and a large proportion of cases were linked to travel to Tunisia (Guerin *et al.*, 2004).

1.9.8 S.Montevideo outbreak caused by contaminated pepper

Furthermore, in 2009 an outbreak of *S.* Montevideo affected 272 individuals from 44 states in the USA, with illness between July 2009 and April 2010. As this affected multiple states, the CDC alongside public health officials developed multiple questionnaires for individuals affected, which contained a list of more than 300 foods and possible exposures during the week of contamination. Investigations revealed that a large proportion of affected individuals had purchased ready to eat salami from a particular company. Subsequent environmental sampling results within the `Wholesome Spice and Seasoning Company' showed the source of the infection was linked to salami products and sealed containers of pepper from one plant. It resulted in the recall of all crushed red pepper sold during the period. Samples were received in state health laboratories where molecular techniques such as pulsed field gel electrophoresis (PGFE) and serotyping were used to identify outbreak strains. The laboratories then forwarded PFGE patterns to PulseNet, which acts as the national molecular subtyping network for foodborne disease surveillance, together they form a crucial network in the surveillance of food borne infections This outbreak is pivotal as there is a common misconception consumers have around the expiry and use of spices in foods, whereas they can actually serve as major vehicles of pathogen transfer (Gieraltowski *et al.*, 2013).

1.9.9 S.Schwarzengrund outbreaks linked to cross contamination with pet food

Moreover, an outbreak of *S.*Schwarzengrund linked to human *Salmonella* infections caused by contaminated dry dog and cat food has been investigated in plants in the United States (Behravesh *et al.*, 2010). Further examination showed that 79 people were affected, of which 48% were children under the age of 2 and illness in infants was associated with feeding pets in the kitchen. A case study was conducted to identify the cause of the outbreak and concluded that infection might have resulted from practices in a limited number of households. Suggesting that organisms multiplied in some households due to cross contamination in the kitchens or pet food bowls not being thoroughly cleaned, both may support bacterial growth. Furthermore infection may have occurred in age groups that were more susceptible to infection with low infectious dose. However, it resulted in a heavy loss for the company who recalled more than 23 000 tons of pet food. An additional outbreak was identified in 2008; the company recalled 105 brands of dry pet food and after three years of human illness being

connected to the plant, they eventually closed the factory down permanently (Behravesh *et al.*, 2010).

These outbreaks highlight the growing need to investigate the source of *Salmonella* contamination and identify how *Salmonella* is able to persist in the factory environment. Microorganisms are able to adhere to food processing surfaces and in the food industry this is clearly problematic. However, routinely factories are swabbed and disinfection protocols are in place to prevent the spread of pathogens. Data from food premises shows that multiple areas within plants are swabbed as part of daily control checks. The Hazard Analysis and Critical Control Points (HACCP) control system, plays an important role in food safety assurance, in addition to programmes that support HACCP, such as the Good Manufacturing Practices (GMPs). A successful factory requires a comprehensive HACCP for the identification of microbial hazards and good GMPs to control microbes present in the factory environment.

1.10 Design of the food manufacturing environment

Microorganisms are able to adhere to food processing surfaces and in the food industry this is clearly problematic. The materials used for machinery and other pieces of equipment used, are not designed with a view to prevent contamination and are often poorly maintained. Floors and walls with cracks, damaged machinery, water pipes and gutters all pose a risk of cross contamination due to the accumulation of microorganisms. As previously mentioned, food factories are not sterile environments and the food factory environment provides a niche for the survival of isolates of *Salmonella* and it has been suggested that some strains become `resident' within the food setting (Habimana *et al.*, 2010a). The growth and accumulation of microorganisms to elevated populations poses a high risk of product contamination. The conditions in the factory environment provide microbes with the essentials for growth, including, time, moisture, nutrients and an adequate temperature. Often organisms accumulate in areas of machinery that are either overlooked or not covered by routine cleaning due to the design of the equipment. The temperatures cycles in the factory are suitable for a range of pathogens and many adapt to temperature fluctuations in addition to low moisture settings (Finn *et al.*, 2013a).

One example of poor microbial management was highlighted in 1985 when an outbreak of *S.* Ealing was associated with dried-milk product from one manufacturer, affecting 70 individuals. The source of contamination was traced back to a factory spray-drier, which had a damaged inner lining; *Salmonella* entered the insulation material from the inner lining and the accumulation of moisture and powder coupled with high temperature allowed the microorganism to grow and eventually contaminate the final product. It resulted in the closure of the plant and the replacement of the equipment (Rowe *et al.*, 1987; Finn *et al.*, 2013a). Furthermore, an investigation into an outbreak of *S.*Infantis linked to dry pet food revealed that equipment in the factory was poorly designed with various cuts and gouges which were difficult to clean and decontaminate, serving as potential sites of *Salmonella* accumulation (Finn *et al.*, 2013a).

Various studies have been previously conducted on surfaces typically found in the feed processing plants, such as stainless steel, granite and plastic (Oliveira *et al.*, 2007; Habimana *et al.*, 2010b). Stainless steel is commonly used in factories as it has many properties which make it an ideal surface, for example it is resistant to corrosion and is protected by a layer of naturally occurring chromium oxide on the surface, which is formed when chromium and air combine. Stainless steel is also inert meaning it does not alter the food products contacting the equipment and it can withstand low and high temperatures, most importantly it is also easy to clean.

Previous studies have investigated how different serotypes of *Salmonella* adhere to surfaces and evaluated the surface hydrophobicity and surface elemental composition (Oliveira *et al.*, 2007). In one study, coupons of steel and polyethylene were immersed in bacterial suspension and their inactivation by biocides commonly used in food industry was investigated. Results showed that *Salmonella* did attach to both surfaces and biocides were not effective in inactivating all the microorganisms adhered on both surfaces (Tondo *et al.*, 2010).

A recent study showed that the persistence of *Salmonella* was correlated to its ability to form biofilms (Vestby *et al.*, 2009b). A biofilm is a complex community of microorganisms that are embedded in a matrix of extracellular polymeric substances. They can attach themselves to both living and inert surfaces (Habimana *et al.*, 2010b). The ability of bacteria to adhere to food contact surfaces depends on various factors, such as the physicochemical properties of the surface of bacterial cells, the hydrophobicity and roughness of the surfaces in the factory.

Bacterial adhesion also relies on the availability of nutrients, the pH and temperature of the medium, cell structures such as flagella and ionic concentration (Oliveira *et al.*, 2007).

Adhesion to glass, steel and polyethylene has been extensively studied, findings indicate that glass and steel formed biofilms more readily than polyethylene (Manijeh *et al.*, 2008). Other studies have investigated the survival and transfer of *Salmonella* Typhimurium from surfaces to food products. It has been shown that *Salmonella* Typhimurium can survive on surfaces for up to four weeks and cross contaminate food almost immediately (Dawson *et al.*, 2007).

Other studies have investigated the survival and transfer of *Salmonella Typhimurium* from surfaces to food products. It has been shown that *Salmonella* Typhimurium can survive on surfaces for up to four weeks and cross contaminate food almost immediately (Dawson *et al.*, 2007). One study investigated the survival of nine factory isolates of *Salmonella* on stainless steel at 23^{0}C. The number of surviving cells were recorded for 30 days and results indicate that *Salmonella* survival was variable and not serotype dependant, whereby the highest survival was observed for *S.* Agona, *S.* Enteritidis, and *S.* Typhimurium DT104 ranging from 4.0 to 4.5 log cfu/ml. whereas an NCTC strain of *S.* Typhimurium showed only 1 log cfu survival after 10 days. This study provides a useful insight into potential growth and survival characteristics of *Salmonella* in dry processing environments and highlight that at temperature close to room temperature *Salmonella* can survive and persist for over a month if surfaces are not disinfected (Margas *et al.*, 2013).

Salmonella have developed strategies to survive in stressed environments such as nutritional deprivation and oxidative stress. Furthermore cells in a biofilm are more resistant to antimicrobial agents than free cells (Paiva *et al.*, 2009). In an investigation determining the efficacy of biosealed for concrete, strains of *Salmonella* were inoculated onto 4 treatment groups, one of which was concrete blocks not treated with the sealant. Internal and external surfaces of concrete blocks were swabbed and plated out onto agar; results proved that the biosealed for concrete was a potent antimicrobial against *Salmonella.*

The use of biocides and disinfectants in the food industry is a key way of controlling environmental microorganisms. Although a range of products are available, food industries select products depending on their compatibility with surfaces such as stainless steel and rubber commonly used for machinery, in addition to their compatibility with the food products being produced. The effective decontamination of surfaces is vital in controlling cross contamination and there are many different classes of antimicrobials used which have specific modes of action against *Salmonella.* The distinct classes of disinfectants include: Quaternary Ammonium Compounds (QACs), hypochlorites, phenolics, aldehydes and alcohol. Each disinfectant class targets specific sites causing metabolic inhibition, membrane disruption and ultimately lysis (Stringfellow *et al.*, 2009).

Cross contamination of food products with *Salmonella* at the factory level is a multifactorial process that is difficult to manage. It involves the management of the organisms entering the food factory on raw materials, the accumulation of organisms which may become `resident', the design of the food factory in terms of equipment and surfaces used, in addition to the role of personnel. Therefore, continued scientific investigations modelling the parameters involved in the spread of pathogens are pivotal in understanding how *Salmonella* is introduced and subsequently survives in food processing environments. In turn this will help inform effective control strategies and the manufacture of safe products.

1.11 Aims and objectives

The aim of this study was to characterise isolates of *Salmonella*, known to be persistent in the food manufacturing environment, by comparing their microbiological characteristics with a panel of matched clinical and veterinary isolates.

Specific objectives were:

- To identify factory environment isolates of *Salmonella* from the Mars culture collection and to create a challenge panel of serotype-matched clinical and veterinary isolates.

- To phenotypically characterise the gross morphology of the challenge panel in terms of cellular size, shape and motility, using well established identification methods including; Scanning Electron Microscopy, API 20E and semisolid media.

- To identify parameters known to influence bacterial survival in the food factory environment; and to define characteristics and environmental factors such as temperature and humidity.

- To investigate if factory strains of *Salmonella* demonstrate an enhanced growth rate in nutrient rich and deficient media.

- To determine the metabolic diversity of the panel using a Biolog Phenotypic Microarray and to analyse complex phenotypic microarray data with Principal Component Analysis (PCA).

- To establish the biofilm formation capacity of the panel at different temperatures and times in both nutrient rich and nutrient deprived media.

- To determine the susceptibility of the panel to a range of disinfectants typically used in the food industry and the ability of these disinfectants to penetrate through biofilms.

- To submit a selection of factory, clinical and veterinary isolates from the panel for whole genome sequencing and to identify any major genomic differences in a panel of candidate genes known to be involved in survival and stress.

2 Chapter 2 phenotypic profiling of isolates to reveal gross morphological differences

2.1 Introduction

Contamination of the food chain with non-typhoidal serotypes of *Salmonella* including both *S.*Typhimurium and *S.* Enteritidis is a significant health concern for both humans and animals (Yang *et al.*, 2002). What is currently not understand is if those isolates that contaminate food processing environments are any different from other isolates of *Salmonella* commonly associated with clinical or veterinary situations, or if they show enhanced environmental adaptation, therefore it is interesting to explore if there are any primary, gross phenotypic morphological differences that can be attributed to strains from a particular environment.

Previous studies have extensively described the use of API20E strips to identify *Salmonella* strains (O'Hara *et al.*, 1993), this is useful as it acts as a control to confirm that the isolates in the collection are actually *Salmonella.* In addition, it provides a limited but descriptive profile of the biochemistry of the organism and in that context it can be used comparatively. Biochemical differences between human and veterinary isolates of *enterobacteriacae* have been studied and marked differences in *Salmonella* biochemical profiles were noted for a few pathways, namely arginine dihydrolase production, citrate utilization, and inositol fermentation (Swanson and Collins, 1980).

2.1.1 The use of scanning electron microscopy

Scanning electron microscopy is a useful tool for the visualisation of the rods to observe how they arrange across a surface and motility is an important factor in pathogenesis and can be observed using semisolid media. Scanning electron microscopy (SEM) was first introduced in the 1950s and is now a common technique used to visualise bacteria that has been fixed, dehydrated, and dried at critical point (Kaláb *et al.*, 2008). As bacteria contain proteins and a high level of water in their cells it is essential to fix them following attachment to preserve their structure before the dehydration step. The SEM uses electrons instead of light to capture an image and has a large depth of field and can capture complex three dimensional structures (Lametschwandtner *et al.*, 1990; Sosinsky *et al.*, 1992; McMullan, 1995).

Scanning Electron Microscopy works by using a beam of electrons to image small structures. The beam of electrons is produced at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final image. For good detection, it is essential the imaging is performed in a vacuum and the surface of the sample is conductive, usually samples are sputter coated with a thin layer of gold before SEM is performed, this also protects the biological sample from becoming damaged. SEM produces images in black if there is zero signal, grey when there are intermediate signals and white in the presence of maximum signal (Warwick, 2010; Iowa-State, 2015). The SEM technique was used to visualise if the factory strains of *Salmonella* demonstrate any differentiating phenotypic characteristics.

Figure 9 A schematic representation of the Scanning Electron Micros (Iowa-State, 2015)

2.1.2 *The use of API20E Microsystems*

Accurately identifying disease causing organisms is essential for clinical microbiology laboratories and phenotyping through the use of API20E Microsystems is a traditional method which has been employed universally since 1970s as an accepted standard (Betancor *et al.*, 2004; O'Hara *et al.*, 1993). Phenotyping is a form of observing the expressed or physical properties of microorganisms such as the presence of flagella, the morphology of the cell wall via Gram staining to differences in metabolic rates and the production of virulence factors (Mistry, 2012). API tests show a biochemical/metabolic profile as microorganisms utilize certain biochemical differently and since they were first introduced the structure and chemical reactions in the strips has not been changed (Darbandi, 2010; Motoshima *et al.*, 2012). In the current study the API 20E (BioMérieux, France) was used as a primary confirmatory test for the positive identification of *Salmonella* and also to identify any primary biochemical differences between the strains in the panel.

2.1.3 *Methods to observe the motility of isolates*

Motility is recognised as a significant biological characteristic and over 90% of *Salmonella* are known to be motile organisms, primarily due to the possession of flagella extending from the plasma membrane and cell wall. Initially detecting motility operated as a mean of bacterial differentiation and classification (Leifson, 1951), Koch's method involved observing microorganisms in a suspended drop of fluid, under a cover slip which held the suspension in place, this method was valuable for observing the shape and arrangement of cells (Klee *et al.*, 2006; Kumar, 2012). This was often used alongside inoculation onto numerous selective media making it a vigorous task. There were many other limitations of the technique including the fact that it was easy to overlook cells as well as the chance of reporting false positive results as motility may be time dependant (Jordan *et al.*, 1934; Tittsler and Sandholzer, 1936). Initially, scientists made up culture media consisting of; beef product, potatoes, gelatine, albumin, and agar. They found that the ingredients combined were useful in detecting motility as results were almost identical to the hanging drop method, following incubation motile organisms grew spreading out from the stab line. However new developments in the isolation media for enteric pathogens supports the rapid growth of the species whilst effectively suppressing non-pathogenic flora (Hine *et al.*, 1988).

In the current study, to observe the motility of the strains in the panel, BBL™ SIM Medium (Oxoid) was used, as it is capable of determining sulphide production, indole formation and motility of enteric microorganisms. The medium contains ferrous ammonium sulphate and sodium thiosulfate, which act as indicators of hydrogen sulphide production; the ferrous ammonium sulphate reacts with H_2S gas and produces a black precipitate called ferrous sulphide; *Salmonella* is detected by the blackening of the medium. Sulfide Indole motility medium also consists of casein peptone, which contains high levels of tryptophan; bacteria that possess the enzyme tryptophanase reduce tryptophan to indole and the addition of Kovacs reagent allows the detection of indole as it couples with p-dimethylaminobenzaldehyde to produce a red band. *Salmonella* is indole negative therefore no band is observed following incubation. The small proportion of agar added to the medium produces a semi solid state which allows motility to be observed. Once the agar is stabbed with *Salmonella,* the organism spreads from the stab line and causes turbidity of the medium which is used as an indication of motility (BD, 2011).

2.1.4 Selection of challenge panel

Routinely, factories are swabbed and disinfection protocols are in place to prevent the spread of pathogens. Data from one factory shown below highlights that within food premises, multiple areas are swabbed as part of daily control checks.

Figure 10 Map to show an example of the multiple sites within a factory that are swabbed daily as part of internal quality control

Figure 10: shows a map of the areas that are routinely swabbed on a daily basis in the Peterborough food factory. Sites include multiple spots in the processing area, mezzanine floor, lifts, walkways, powder area, office area, inside machines and wet bins. The organisms identified in each area can be seen in figure 11.

The swabs are processed and confirmed in the laboratory and positive swab results are recorded for each area of the factory. This helps reveal any serotype trends in isolated data for each location.

Figure 11 Swab data revealing *Salmonella* isolated from each zone within a factory site

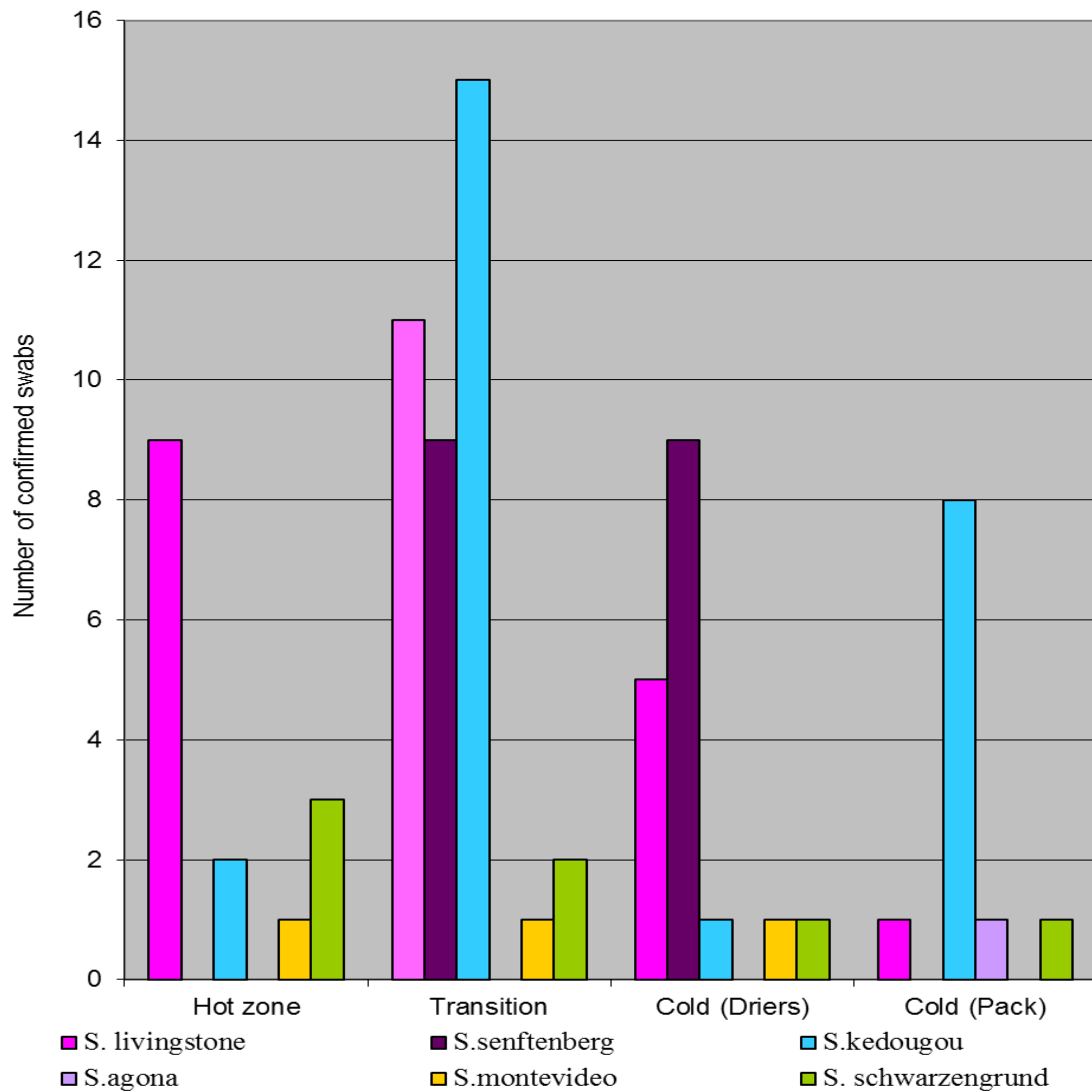


Figure 11: shows confirmed swab data from the food factory shown in figure 10, it reveals a distinctive pattern in the serotype of strains that were isolated in all four zones of the factory; in the hot, transition, drying and packing zone of the factory.

2.1.5 *Creation of challenge panel of isolates*

Based on the swab data, a small panel of challenge *Salmonella* isolates was created, which consisted of ten isolates of *Salmonella* and the NCTC strain of *L.monocytogenes* (11994). As Table 3 shows, the *Salmonella* isolates represented veterinary, clinical and factory environments and the following serotypes were included; *S.*Schwarzengrund, *S.*Senftenberg, *S.*Montevideo, *S.*Livingstone, *S.*Kedougou and *S.*Typhimurium.

Table 3 Challenge panel of isolates

Strain	Source
***S*.Senftenberg 775W**	ATCC 43845
***S*.Senftenberg**	PBO factory
***S*.Senftenberg**	VLA
***S*.Schwarzengrund**	FSL S5-458 American clinical
***S*.Schwarzengrund**	USA factory
***S*.Schwarzengrund**	VLA
***S*.Typhimurium SL1344**	NCTC 13347
L. monocytogenes	NCTC 11994
***S*.Livingstone**	PBO factory
***S*.Kedougou**	PBO factory
***S*.Montevideo**	USA factory

Table 3: shows the final panel of isolates selected for a majority of the investigations alongside the origin of the isolates. Serotype-matched clinical and veterinary isolates were sourced for the factory isolates of *S.*Senftenberg and *S.*Schwarzengrund in an attempt to balance serotypes.

The factory isolates originated from the Mars Peterborough (UK) and the USA pet food care plant and these are abbreviated as PBO and USA. The *S.*Schwarzengrund and *S.*Senftenberg factory isolates were serotype matched to clinical and veterinary isolates, so that any potential significant differences could be differentiated as an effect of environment rather than serotype. The heat resistant strain *S.*Senftenberg 775W is well-documented and unlike other strains it is a non-hydrogen sulphide producer. Globally, S.Senftenberg is not a major cause of salmonellosis and outbreaks are commonly associated with contaminated poultry and plant derivatived food. Yet antimicrobial resistance data suggests that surveillance of this serotype is important. Typically in laboratories, the production of hydrogen sulphide on selective media is used as a presumptive test for the identification of *Salmonella.* The lack of H2S production of *S.*Senftenberg has been documented in previous literature but not extensively (Henry *et al.*, 1969; Yi *et al.*, 2014). A surveillance report from China between 2005 to 2011, shows that 8.4% of *Salmonella* isolates recovered from human stool, animal, food and environmental samples were identified as *S.* Senftenberg and of these 9.3% were non H_2S-producing (Ng *et al.*, 1969; Yi *et al.*, 2014). *S.*Schwarzengrund USA caused an outbreak associated with pet food in 2009 and the *S.*Schwarzengrund (FSL S5-458) is the American clinical isolate which was isolated from patients during the outbreak (Behravesh *et al.*, 2010). *S.*Typhimurium SL1344 has been typed and literature shows that the serotypes Typhimurium and Enteritidis are the leading cause of *Salmonella* disease (Vestby *et al.*, 2009b; Msefula *et al.*, 2012). *L.monocytogenes* (11994) was added to the panel as a control, as it is recognised as a persistent strain in food factories and is capable of forming strong biofilms (Lee Wong, 1998).

The aim of the work described in this chapter was to phenotypically characterise isolates of *Salmonella* from factory, clinical and veterinary environments, in order to determine any gross morphological differences characteristic of the strains from each environment.

2.2 *Materials and Methods*

2.2.1 *Microbiological media*

Nutrient Agar and Nutrient Broth were purchased from Oxoid (Basingstoke, UK) and sterile Phosphate Buffer Saline (PBS) was purchased from Fisher Scientific, UK. The aforementioned media were prepared as per manufacturer's instructions and sterilised by autoclaving at 121°C for 15 minutes. The PBS was stored at 4°C until required. BBL™ Sulfide Indole Motility Medium for observing motility was pre-made and was purchased from Oxoid (Basingstoke, UK).

2.2.2 *Microbial cultures*

All the isolates in the challenge panel were stored on micro bank beads (Fisher Scientific) at -80°C until required, a bead of each isolate was recovered from frozen storage into 5ml of Nutrient broth and grown at 37°C or 24 hours.

2.2.3 *Scanning electron microscopy*

In order to allow the bacteria to colonise the sample, thermanox plastic cover slip discs (Fisher Scientific) 1 cm^2 were immersed in 2mls of nutrient broth containing a 10^6 suspension of bacteria and grown at 37°C at 100rmp in an orbital shaker for 24 hours. After 24hours the media was removed and replaced with 2ml of fresh nutrient broth and incubated for a further 24 hours. Following incubation the discs were rinsed with PBS to remove any culture medium. The discs were then fixed for 15 minutes at room temperature in freshly prepared glutaraldehyde cacodylate buffer (2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2-7.4). Next, the samples were dehydrated through an ethanol series of 20% to 100% in 10% increments for 10 minutes each up to 90% ethanol, after which the samples were dehydrated with 95% and 100% and both steps were repeated twice, ensuring the samples did not dry out at any point. The samples were then critical point dried using hexamethyldisilizane (HMDS) in a fume cupboard to remove final alcohol. For this final step the discs were placed into a 24 well micro titre plate, the last traces of ethanol removed using a pipette and the well flooded with HMDS to completely cover the sample and incubated overnight in a fume cupboard. The coupons were then sputter coated with gold and examined using

a EISS EVO® MA and LS Series Scanning Electron Microscope (SEM). The sample was focussed and the bacterial rods were measured using the Auto smart software linked to the microscope. Initial experiments were performed using stainless steel discs however the images were difficult to focus and resolve (Figure 12). An Analysis of Variance (ANOVA) was conducted in Statistica (version 10, USA), to reveal any potential differences in rod length across the strains and the three environments. The Scanning electron microscopy images of *Salmonella* and *L.monocytogenes* cells attached to thermaonox coverslips after 48 hours in nutrient media are shown in Figures 13-19. Images were captured using a Zeiss Evo10MA Scanning Electron Microscope at 10 – 20 Kv and a working distance of 6 – 10mm, at magnifications ranging from 300x – 5000x. Scale bars are shown on the individual images. The images were taken at 20 µm, 10µm and 2 µm.

2.2.4 *Motility Test*

The motility of the panel of *Salmonella* isolates was determined by performing a motility test. Isolates stored at -80°C were resuscitated, a bead from each culture was removed and the vial was put back into the freezer so that the remainder of the culture did not thaw. The bead was inoculated onto nutrient agar (NA) plates and incubated at 37°C for 24 hours. The BBL™ SIM Medium was purchased from Oxoid (Basingstoke, UK), following incubation a single colony, WAS taken from the NA plate and was used to inoculate the SIM media by stabbing the centre of the agar tube in a vertical direction. Inoculated test-tubes were incubated at 37°C for 24 hours. *Salmonella* was detected by blackening of the media and motility was defined as horizontally dispersed growth from the stab line (Shields and Cathcart, 2013).

2.2.5 *Biochemical profiling using API 20E*

The API 20E (BioMérieux, France) was used as a primary confirmatory test for the positive identification of *Salmonella* and also to identify any biochemical differences between the strains in the panel. Suspensions of each strain were prepared in 5mL volumes of sterile 0.85% (w/v) saline to a 0.5 MacFarland standard. API 20E strips were inoculated and analysed in line with the manufacturer's instructions. The plastic strips consisting of twenty mini-test tubes were inoculated with the bacterial suspension; ensuring air bubbles were not introduced. Some of the tubes were filled to

the top (CIT, VP and GEL), whereas other tubes were overlaid with mineral oil to allow anaerobic reactions to take place (ADH, LDC, ODC, H2S, URE). The API strips were then placed in sterile plastic containers containing 5mls of sterile water to uphold humidity and incubated at 37°C for 18-24 hours. Following incubation the colour change in the strips was read and interpreted according to the manufacturer's guidelines. The negative control was inoculated with saline and reactions were denoted positive or negative depending on the appropriate colour change (Imen *et al.*, 2012; Mistry, 2012). *Salmonella* reactions are further described in table 4.

2.2.6 Data analysis

The API profiles of the *Salmonella* isolates were examined using the internet identification tool ApiWeb database (BioMérieux, France). The database allows for the identification of bacteria at a genus and species level following a 24 hour incubation time. The 20 biochemical reactions on each API strip, following incubation with each *Salmonella* isolate, were coded as positive or negative respectively depending on the colour change, the results were then entered into the data base to provide identification. The SEM images were analysed visually and the arrangement of the rods across the discs was observed. From this 15 rods were measured using the tool on the Axio programme and the average *Salmonella* and *L.monocytogenes* rod length was calculated for each strain. Further statistical analysis was conducted via a One way Analysis of variance (ANOVA) using Statistica software as it allows comparison of any differences across mean cell lengths between the strains from the three environments.

2.3 Results

Bacterial isolates were visualised under SEM and the length of the rods calculated using associated image analysis software. Images were prepared of bacterial cells attached to thermanox coverslips, and the length of the rods calculated using associated image analysis software

Figure 12 *Salmonella* cells on stainless steel discs.

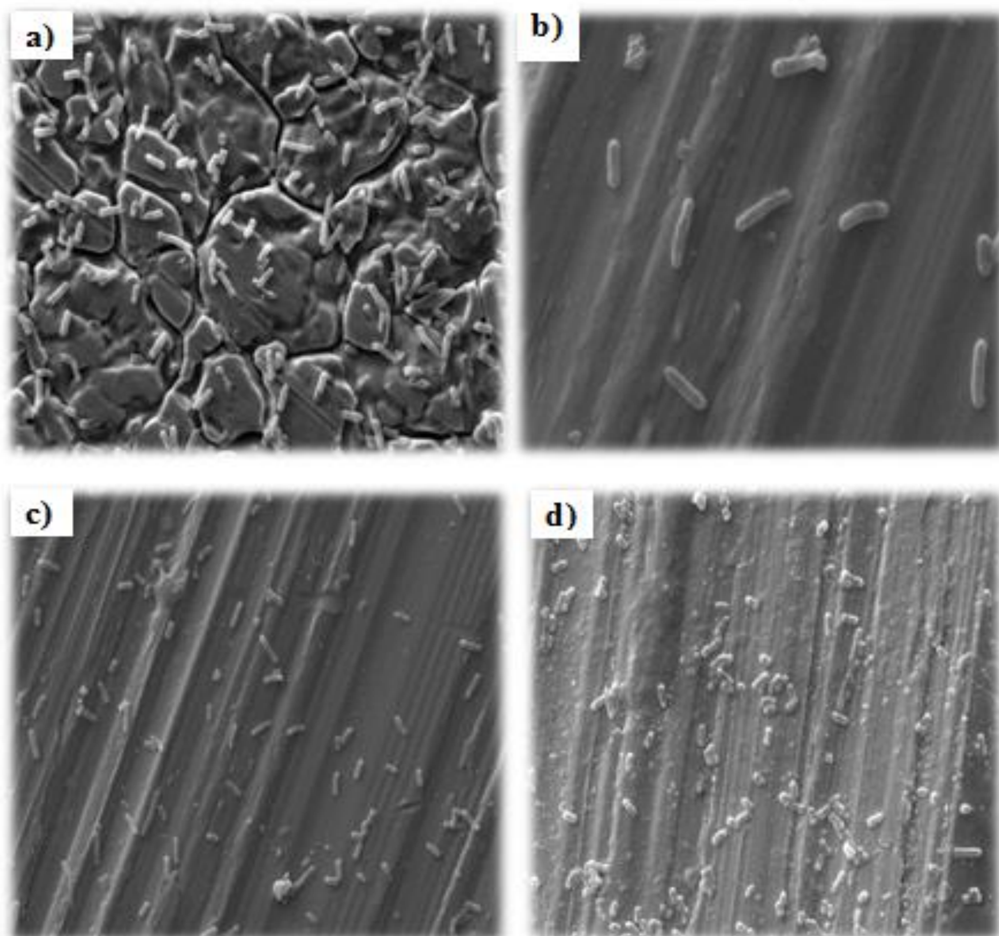


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Figure 12 a-d: SEM images of *Salmonella* attached to stainless steel coupons a) *S*.Senftenberg clinical, b) *S*.Senftenberg vet c) *S*.Senftenberg factory & d) *S*.Montevideo factory. Initially *Salmonella* cells were attached to 1cm^2 stainless steel discs; however the natural roughness and appearance of the discs proved a difficult background to visualise the cells. Images were captured at 2µm and show that *Salmonella* rods do attach to stainless steel however it is challenging to make any clear conclusions on rod size and length. Therefore in further experiments, thermaonnox plastic cover slips were used as they have a smoother appearance and allow distinct images to be captured.

Figure 13 SEM images of the factory isolate *S.*Montevideo attached to thermanox cover slips

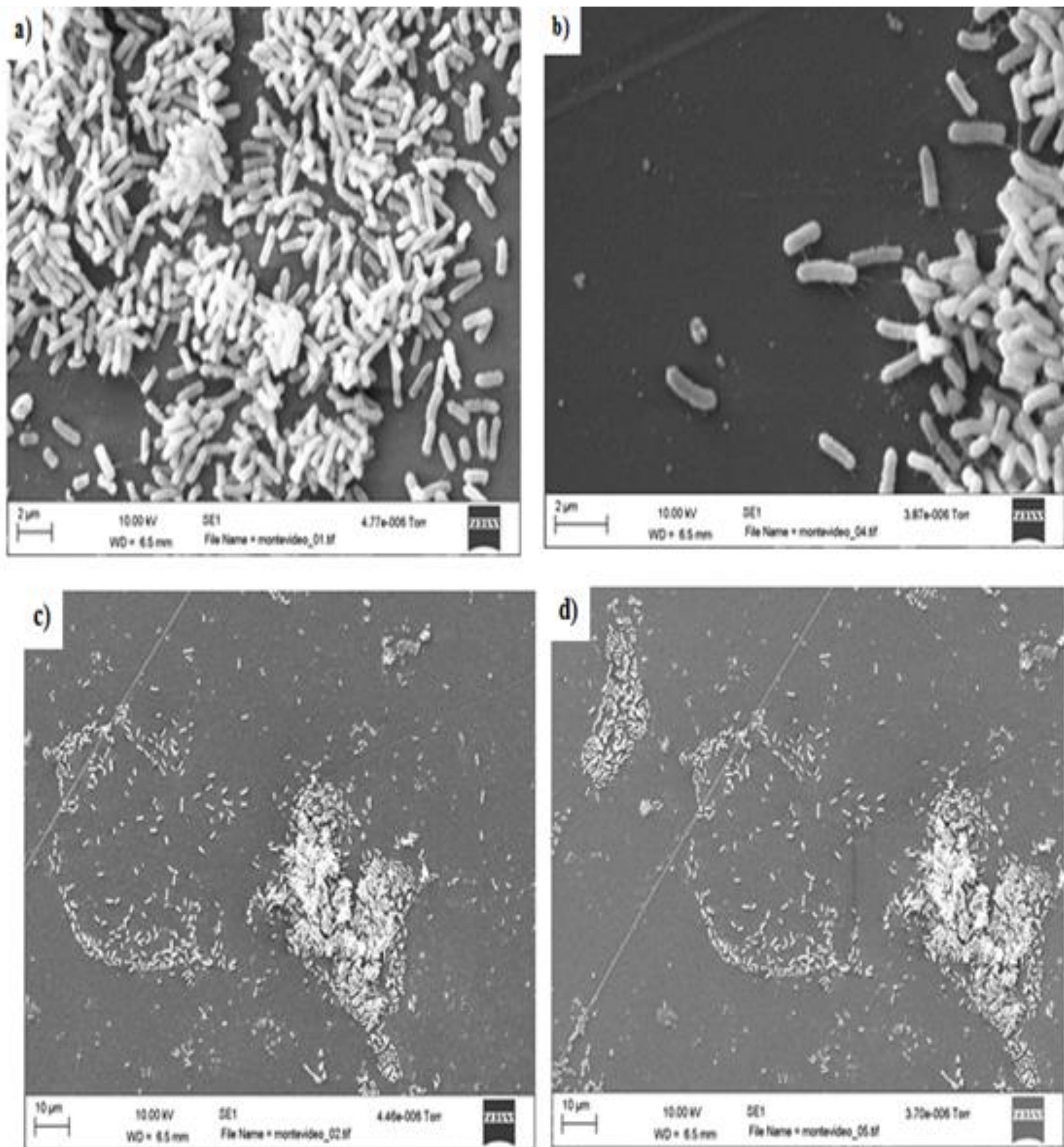


Figure 13 a-d: SEM images of the factory isolate of *Salmonella* Montevideo on plastic thermanox cover slips. 13 a-b) captured at a 2µm distance, with b) showing cells at the edge of the disc. Small surface appendages can be seen projecting from the rods, these structures may be involved in aiding in attachment to the surface and each other, the cells are overlapping and the stacks of rods appear to be arranged in rafts 13 c-d) images taken at a 10µmdistance and a distinct cluster of cells can be visualised.

Figure 14 SEM images of *S*.Livingstone factory attached to thermanox cover slips

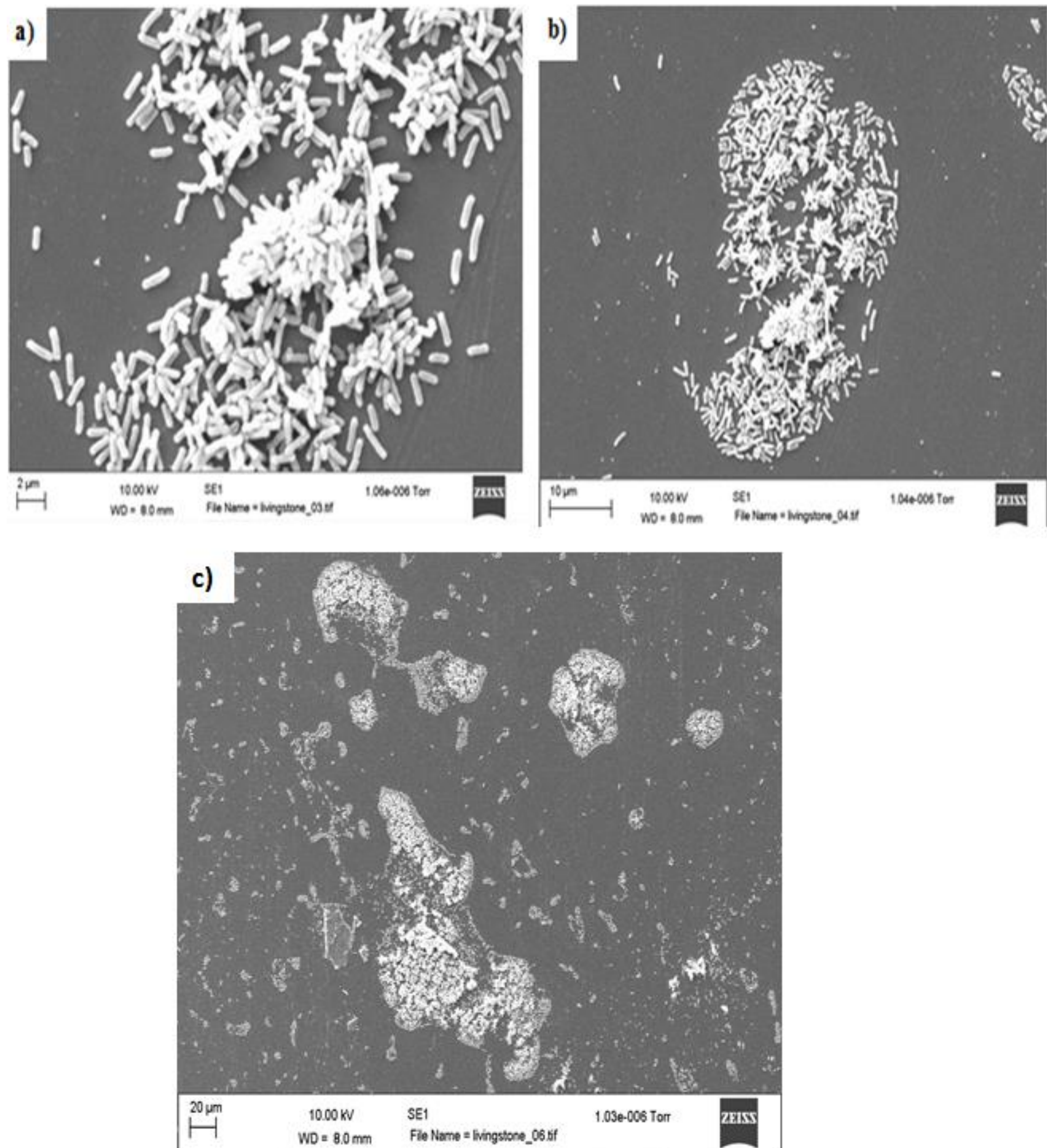


Figure 14 a-b: SEM images of the factory isolate of *Salmonella* Livingstone on plastic thermanox cover slips a) was captured at 2µm showing the cells in the cluster magnified, whereas b) was captured at 10µm and shows a cluster of rods all tightly arranged in a dense area. 14c: image captured at 20 µm showing an overview of how the cells were arranged across the disc.

Figure 15 SEM images of the clinical isolate of *S.*Schwarzengrund attached to thermanox cover slips

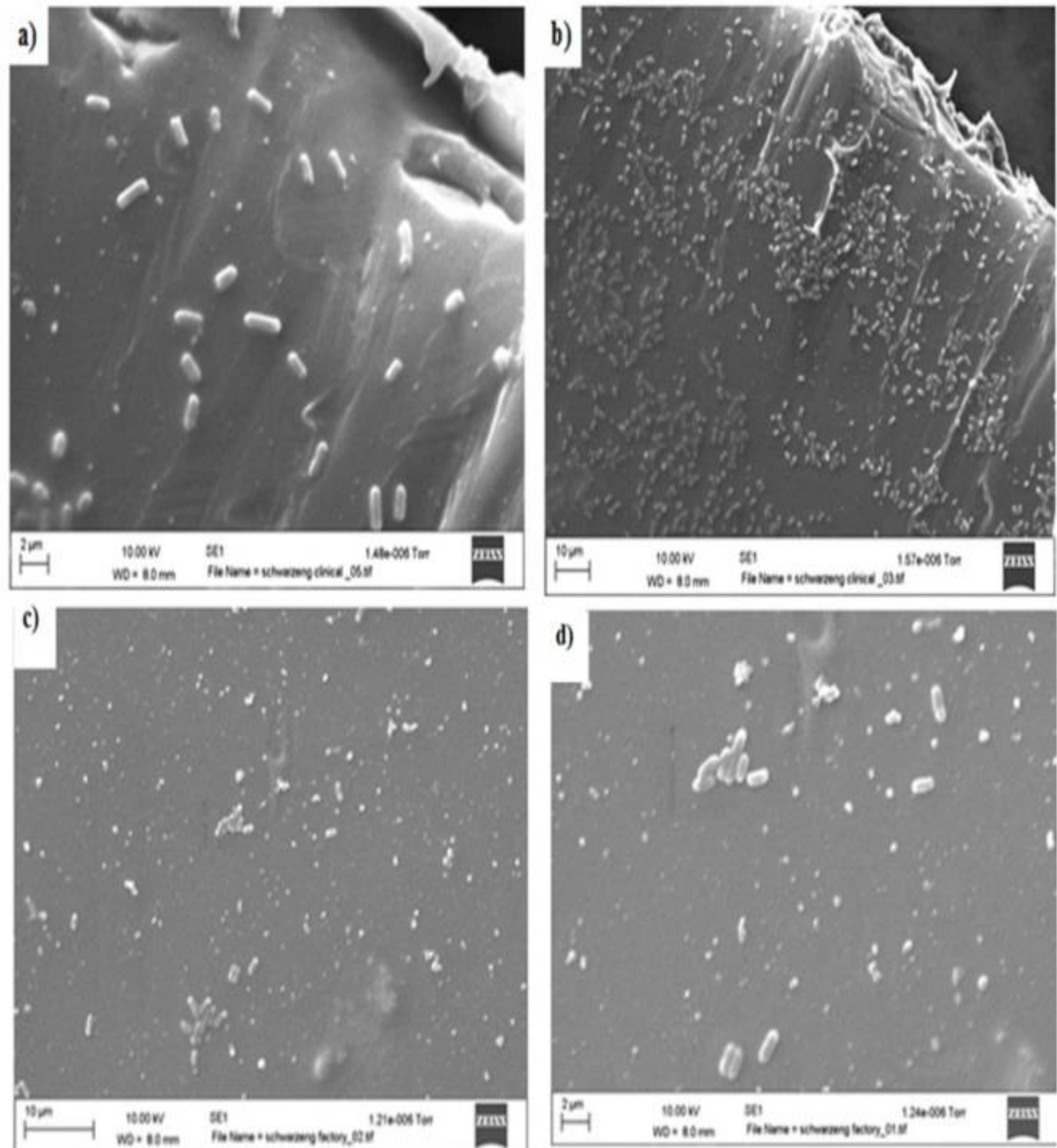


Figure 15 a-b: SEM images of the clinical isolate of *Salmonella* Schwarzengrund a) were captured at 2µm showing individual rods whereas b) was captured at 10µm and shows a cluster of rods across the disc. c-d) images of the factory isolate of *Salmonella* Schwarzengrund c) was captured at 10µm showing a few individual rods whereas d) was captured at 2µm and shows few rods scarcely spread across the disc.

Figure 16 SEM images of the veterinary isolate of *S*.Schwarzengrund attached to thermanox cover slips

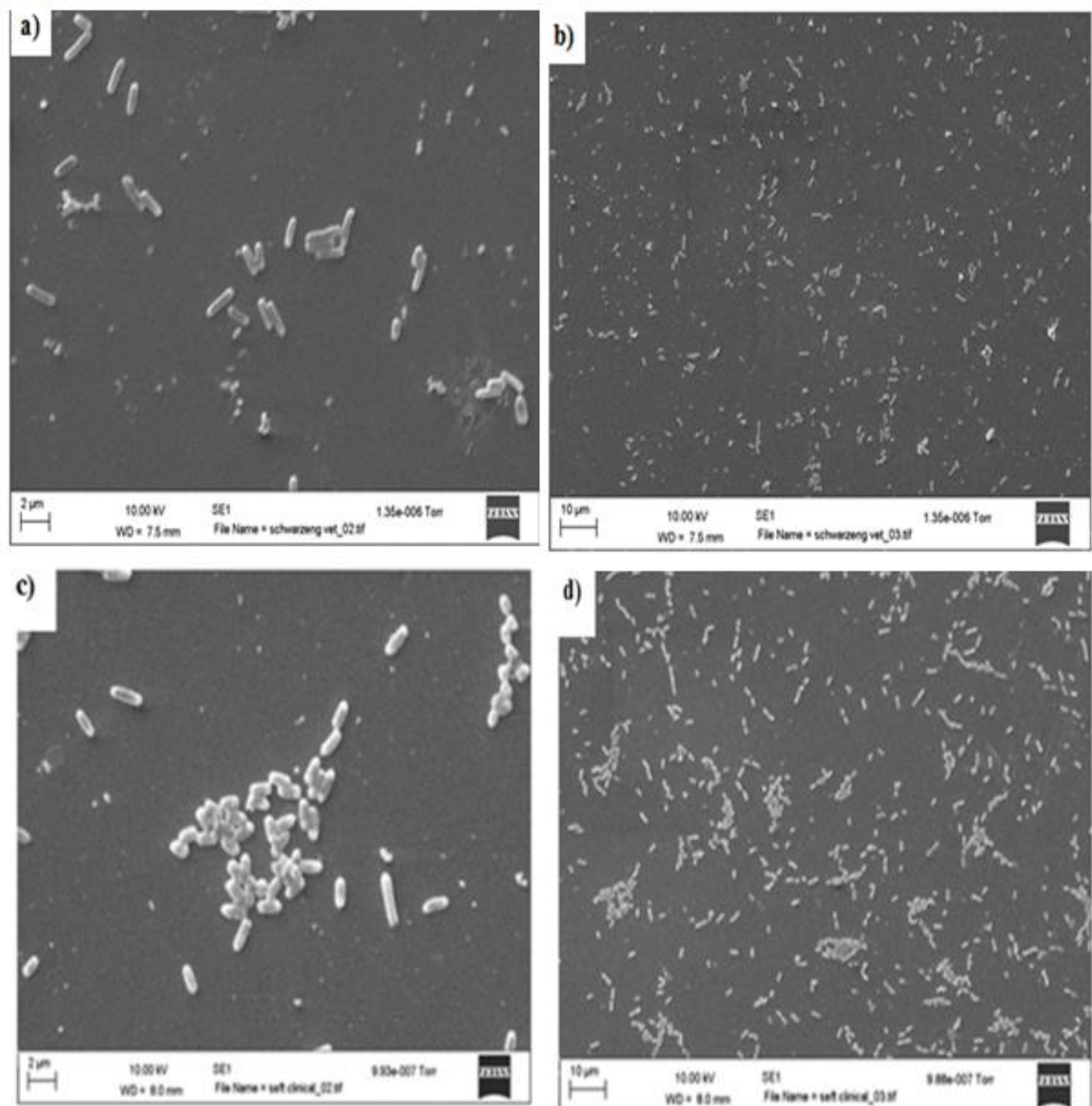


Figure 16 a-b: SEM images of the veterinary isolate of *Salmonella* Schwarzengrund a) was captured at 2µm showing individual rods whereas b) was captured at 10µm and shows the rods scattered across the disc but not forming any distinct cluster. c-d: SEM images of the clinical isolate of *Salmonella* Seftenburg c) were captured at 2µm showing individual rods whereas d) was captured at 10µm. The cells are arranged in small clusters

Figure 17 SEM images of the factory isolate of *S*. Senftenberg attached to thermanox cover slips

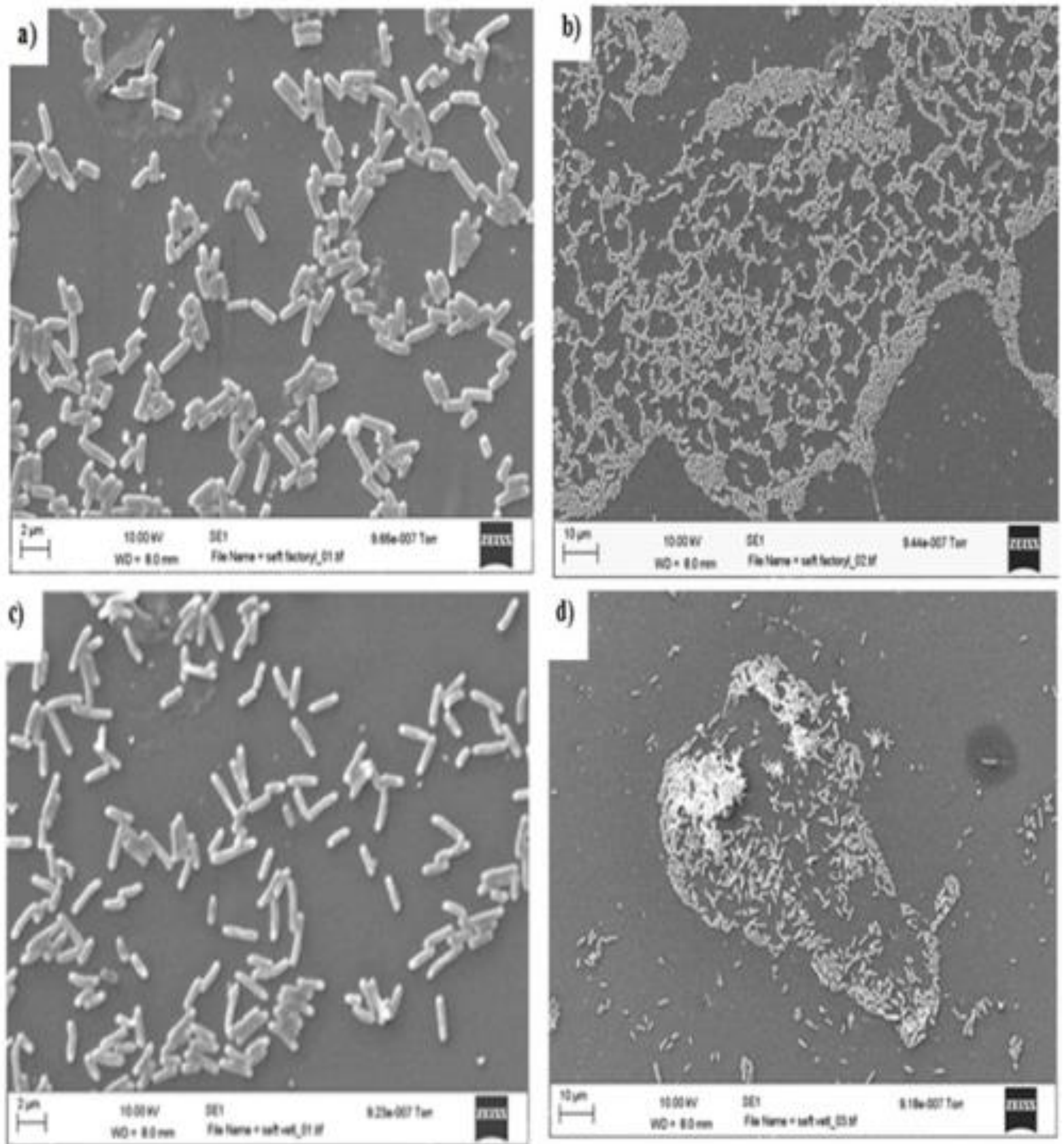


Figure 17 a-b: SEM images of the factory isolate of *Salmonella* Senftenberg a) was captured at 2µm showing rods attaching to one another and forming clusters b) was captured at 10 µm and shows a distinct clustering pattern, with the rods arranged in tight pattern c) was captured at 2µm showing the cells in the cluster magnified, whereas d) was captured at 10µm and shows a cluster of rods all tightly arranged and an area here the cells are overlapping densely.

Figure 18 SEM images of *Salmonella* Typhimurium SL1344 attached to thermanox cover slips

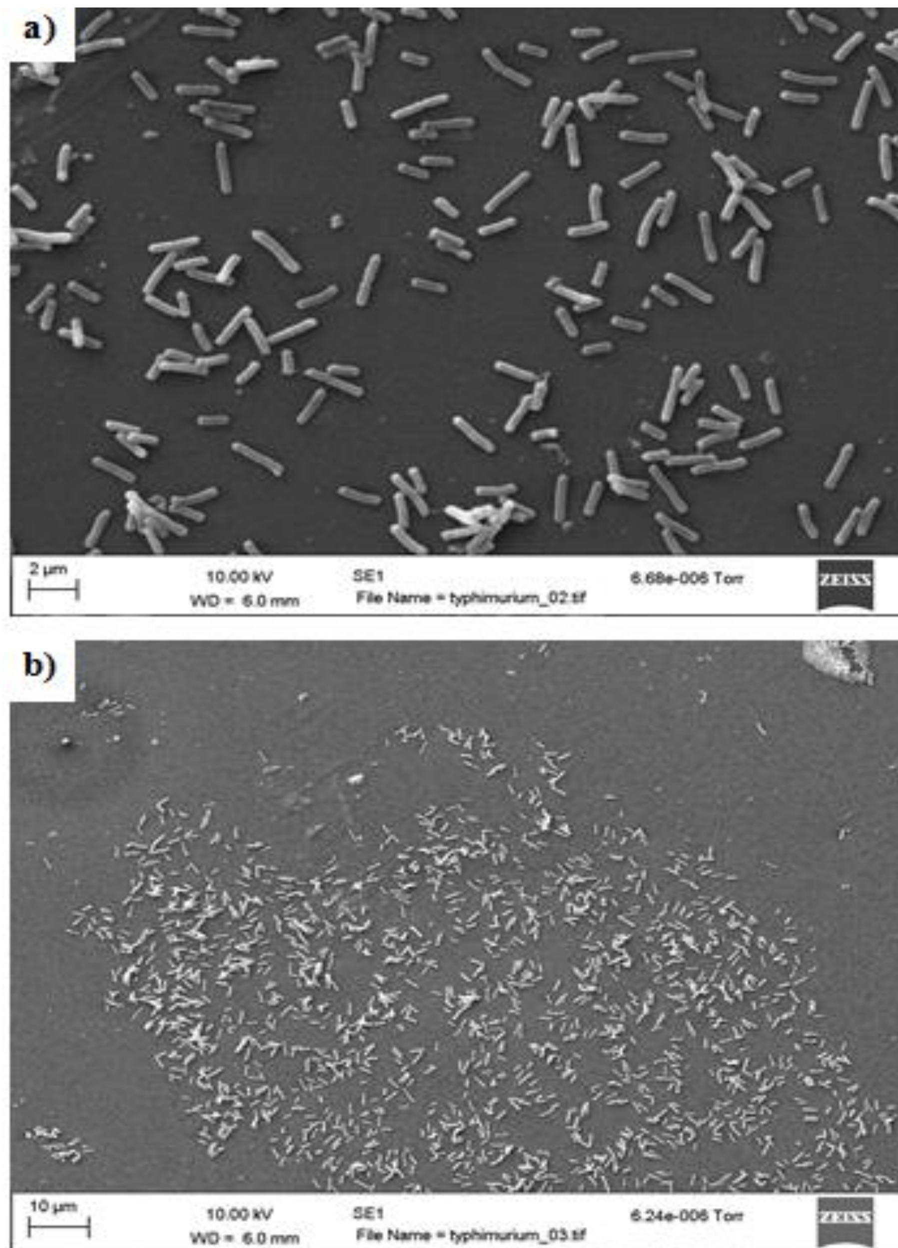


Figure 18 a-b: SEM images of *Salmonella* Typhimurium SL1344 on plastic thermanox cover slips a) was captured at 2µm showing the cells in the cluster magnified, whereas b) was captured at 10µm

Figure 19 SEM images of *L.monocytogenes* attached to thermanox cover slips

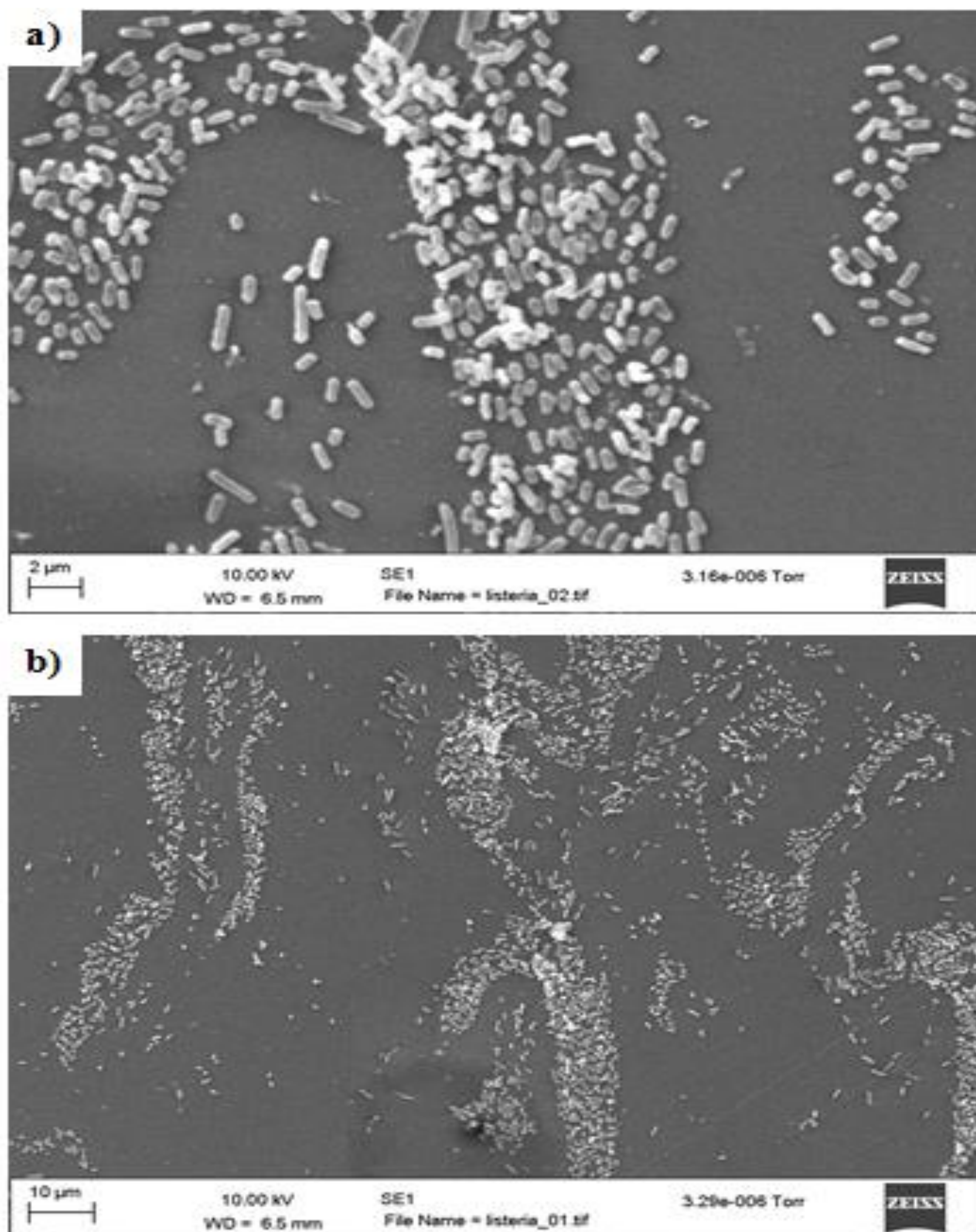


Figure 19 a-b: SEM images of *Listeria monocytogenes* on plastic thermanox cover slips a) was captured at 2µm showing the cells in the cluster magnified, whereas b) was captured at 10µm.

The length of 15 rods within a field of view was calculated using image capture software and the length in µm recorded as shown in figure 20.

Figure 20 Histogram to show comparison of rod length measured using SEM

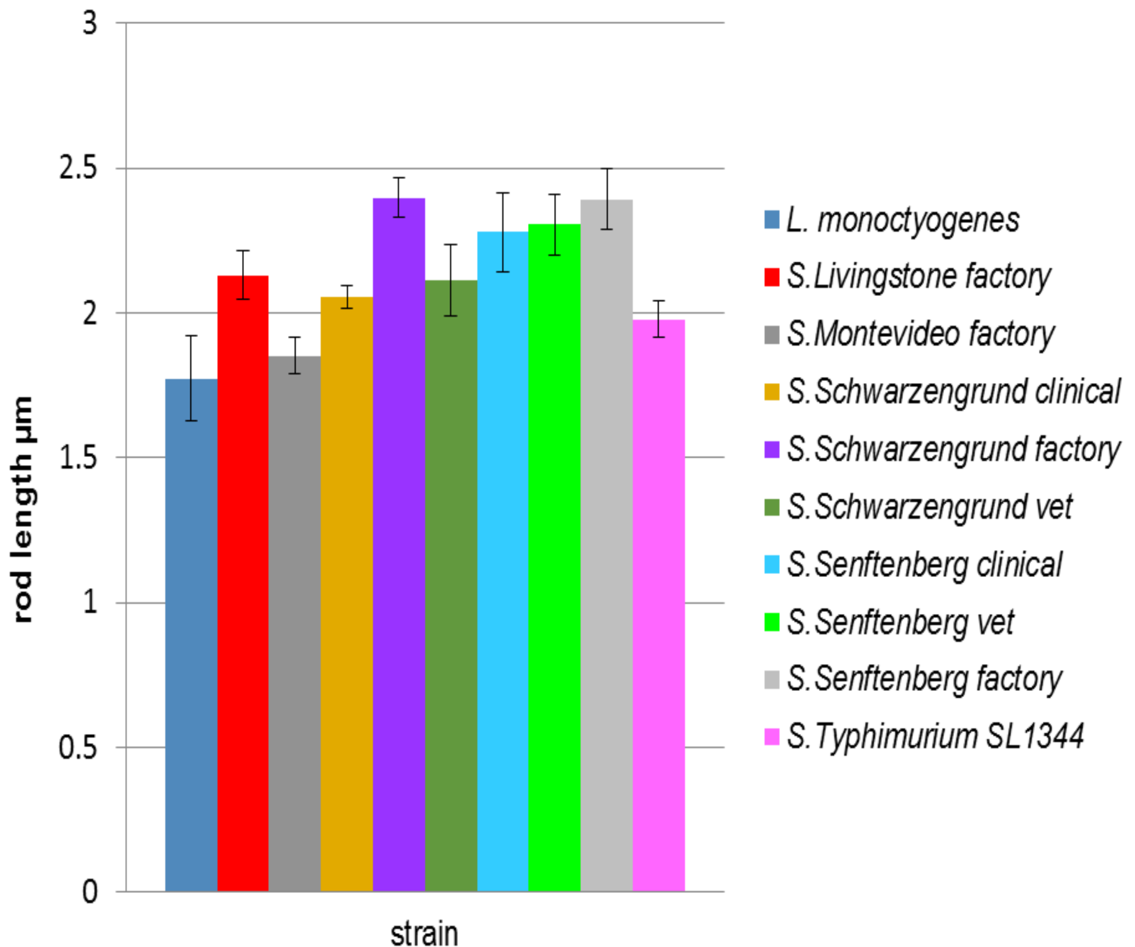


Figure 20: Average *Salmonella* and *L.monocytogenes* rod length. From the Scanning Electron Microscopy (SEM) images 15 rods were measured to determine the average *Salmonella* and *Listeria monocytogenes* rod length. The error bars represent standard deviation, the average rod length ranged between 1.77µm to 2.39µm. Further statistical analysis was conducted to determine if the variance in rod length was significant.

The rod lengths were analysed using an ANOVA to determine if there was any significant difference between the rod lengths of the different serotypes of *Salmonella*. The output from STATISTICA (version 10, USA) can be seen in Figure 21.

Figure 21 ANOVA to reveal comparisons in mean rod lengths for the different *Salmonella* serotypes

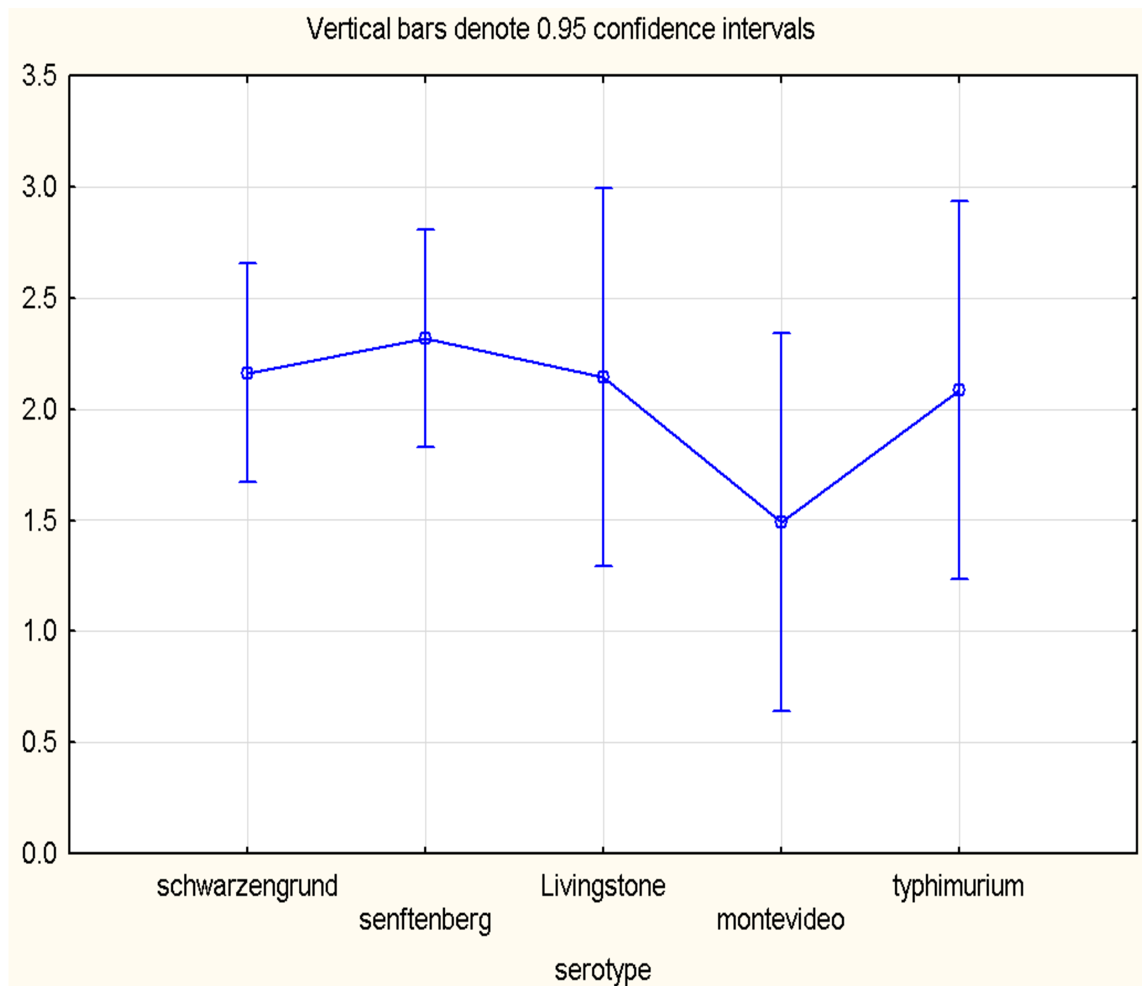


Figure 21: The output from the ANOVA for the different serotypes of *Salmonella*. Using the data generated from measuring the rod lengths from the SEM images, Analysis of Variance (ANOVA) was conducted to highlight any potential difference in rod length across the strains. The results indicate there was no significant difference in gross phenotype (p=0.343) across the serotype.

Further statistical analysis was undertaken to determine whether the environment from which the *Salmonella* strains were isolated influenced the length of the bacterial cell. These data are shown in figure 22.

Figure 22 The effect of environment on bacterial cell length

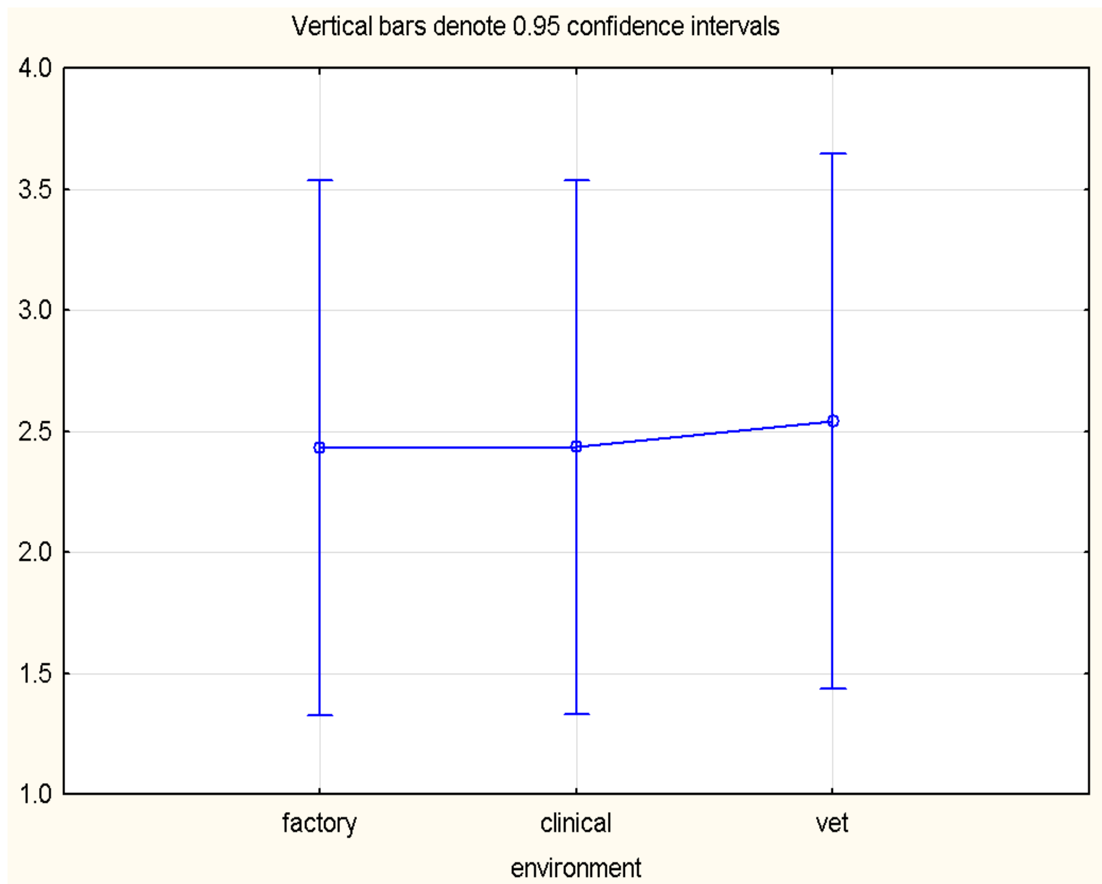


Figure 22 the output from the ANOVA test investigating differences in environment. Further analysis shows that the environment of the strains does not influence rod length. There is no significant difference mean bacterial rod length across clinical, veterinary and factory strains of *Salmonella* (p=.389).

2.4 Biochemical profiling using API 20E

All isolates were subject to biochemical profiling using API 20E strips. A representative result of an APiI20E strip positive for *Salmonella* is shown in figure 23.

Figure 23 A positive results for *Salmonella* using API20E test

Figure 23: Typical *Salmonella* positive result with API 20E strip following incubation (Imen *et al.*, 2012). Following inoculation with a bacterial suspension in saline, the *Salmonella* isolates metabolize the substrates during the overnight incubation and develop a colour change. The reactions that occur in the API strip are identical to the established macro-methods.

Table 4 List of the biochemical reactions in an API 20E kit

Test	Substrate	Reaction	Negative result	Positive result	*Salmonella* spp
ONPG	ONPG	Betagalactosidase	colourless	yellow	-
ADH	arginine	Arginine dihydrolase	yellow	Red /orange	-
LDC	lysine	Lysine decarboxylate	yellow	Red/ orange	+
ODC	ornithine	Ornithine decarboxylate	yellow	Red/orange	+
CIT	citrate	Citrate utilization	Pale to green/yellow	Blue-green/blue	-
H2S	NA thiosulfate	H2S production	Colourless/grey	Black deposit	+
URE	urea	Urea hydrolysis	yellow	Red/orange	-
TDA	tryptophan	deaminase	yellow	Brown- red	-
IND	tryptophan	Indole production	yellow	Red in 2min	-
VP	Na pyruvate	Acetoin production	colourless	Pink.red in 10m	-
GEL	Charcoal gelatin	geltinase	No diffusion of black/ blue	yellow	-
GLU	glucose	Fermentation/ oxidation	Blue- green blue	yellow	+
MAN	Mannitol	Fermentation/ oxidation	Blue- green blue	yellow	+
INO	Inositol	Fermentation/ oxidation	Blue- green blue	yellow	-
SOR	Sorbitol	Fermentation/ oxidation	Blue- green blue	yellow	+
RHA	Rhamnose	Fermentation/ oxidation	Blue- green blue	yellow	+
SAC	Sucrose	Fermentation/ oxidation	Blue- green blue	yellow	-
MEL	Melibiose	Fermentation/ oxidation	Blue- green blue	yellow	+
AMU	Amygdalin	Fermentation/ oxidation	Blue- green blue	yellow	-
ARA	Arabinose	Fermentation/ oxidation	Blue- green blue	yellow	+

Table 4: a list of the biochemical reactions in the API 20E, the last column shows the typical *Salmonella* positive reactions observed. With the exception of *S.*Senftenberg 775W, this was H_2S negative. The table is adapted from Imen *et al.* (2012). The profile built from the final column was entered into the online system to provide a match to *Salmonella* spp.

2.5 Motility testing

All isolates were tested for motility using SIM media and the results are shown in Figure 24.

Figure 24 Detecting motility and the presence of hydrogen sulphide in *Salmonella* using SIM media

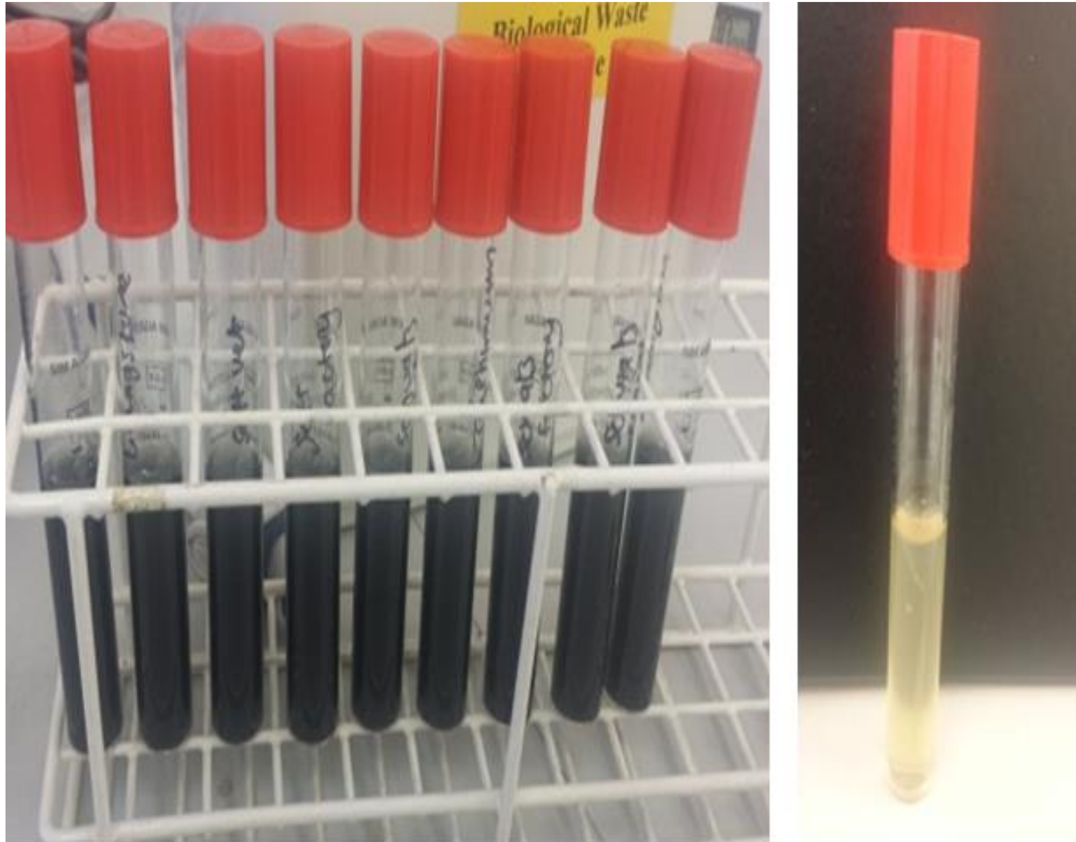


Figure 24: The nine tubes on the left show that the *Salmonella* isolates produced a black precipitate of ferrous ammonium sulphate that reacted with H_2S gas, this is a typical observation used for the identification of *Salmonella* and motility. The tube on the right shows *S*.Senftenberg 775W, which is known to be H_2S negative, therefore the colour change is not observed but motility was observed via the turbidity in the tube and spreading of growth from the stab line.

2.6 *Discussion*

The aim of this chapter was to profile, phenotypically a panel of factory, clinical and veterinary isolates using API 20E, motility testing and SEM. Previous studies have shown that *Salmonella* strains differ in their metabolic characteristics and these differences may expose differences across strains (Lewis and Stocker, 1971).

Scanning electron microscopy is a powerful imaging technique that can magnify and resolve images with sufficient clarity and power that the length of the cells can be accurately measured. In the current study the technique was utilised to investigate the gross morphology of *Salmonella* strains from factory, veterinary and clinical environments. It was observed that none of the isolates showed any significant difference in the length of the isolates when compared. This was from different strains from different environments and therefore it can be concluded that there is no gross morphological difference within these strains. The average length ranged from between 1.85µm to 2.39µm and this fits exactly within the ranges that may be expected. Both Harshey & Matsuyama (1994) and Motarjemi (2013) also described the length of *Salmonella* within 2-4 micrometres, therefore the lengths measured in this study are entirely consistent with the expected size of *Salmonella* (Harshey and Matsuyama, 1994; Motarjemi, 2013; Andino and Hanning, 2015). The *L.monocytogenes* rods were slightly shorter in length, with an average length of 1.77 µm in comparison to *Salmonella* but this was as expected as the average cell length of *L.monocytogenes* ranges from as little as 0.5µm to 2.0µm (Batt and Robinson, 1999; Miliotis and Bier, 2003).

Bacterial shape holds biological relevance and although there are an array of shapes including cocci, rods and spirochetes and microorganisms which may arrange in chains or clumps, bacteria only display a limited subset of morphologies, which indicates that microorganisms only embrace a shape that is adaptive. Furthermore in 1961, a study showed that when grown in nutrient-limiting conditions, streptococci grew as true filaments instead of the typical cocci arrangement (Frehel *et al.*, 1988; Young, 2007), indicating that bacteria can change shape depending on the environment. There are other examples within other bacterial species whereby cell shape changes as a result of environmental stress, for example *Campylobacter* changes from a slim spiral or curved shaped cell to a coccoid shaped cell under certain conditions of stress such as prolonged culture or with temperature changes (Jang *et al.*, 2007). It is unknown if this change is degenerative or a functional change.

Previously, Mattick *et al.* (2000) investigated the morphological changes in isolates of *S. enterica* serovar Enteriditis PT4 and *S. enterica* serovar Typhimurium DT104 at reduced A_w values and revealed that the *Salmonella* strains tested formed filaments at both 21°C and 37°C. The presence of filamentation in low-A_w food products could pose serious implications for the food industry as contaminant *Salmonella* cells may continue to replicate and lead to an increase in biomass in addition to a build-up of these long filaments in foods. This is problematic as the testing of low A_w foods via traditional microbiological methods such as direct plating or enrichment would reveal a low count. However under favourable conditions, for example if the -a_w increases of foods through rehydration with water, septation will occur and give presence to a large number of viable *Salmonella* cells, which can cause infection following consumption (Mattick *et al.*, 2000).

Therefore the shape of the isolates in the panel was investigated from a gross morphological point of view as a possible indicator of difference within the group; to reveal if *Salmonella* from the environment or from stressed conditions was able to change shape or size.

Interestingly, for the factory strain of *S.*Livingstone, *S.*Montevideo and the veterinary isolate of *S.*Senftenberg in some of the images small surface appendages protruding from the rods can be visualised, these are almost acting as a mechanism of adhesion from one cell to another. However one of the limitations of this technique is that sputter coating the fixed and dried bacteria with a 20 nm thick layer gold obscures fine structures such as pili and fimbriae, therefore to further explore these structures, techniques such as transmission electron microscopy (TEM) could be used (Perfumo *et al.*, 2014; Tang *et al.*, 2014). These surfaces appendages may have a key role in adhesion to cells and surface which is advantageous in processes such as biofilm formation (Rai and Bai, 2014).

In the current study, motility was observed through the use of a semi-solid medium. This medium can act as a primary diagnostic tool when looking at differences between and within strains. Of the 2500 serovars of *Salmonella* a majority are motile; this suggests that motility may be implicated in infection. In the study all the isolates of *Salmonella* from clinical, veterinary and factory environments demonstrated motility. The extra animal stage of *Salmonella* infection is important in the full infection cycle of zoonosis in *Salmonella*, one study investigated the role of flagella in this stage in *S.*Typhimurium and *S.*Dublin and found that motility played a minor role in the spread

of *Salmonella* from one animal to the next through the external environment, however it is important to note that this is only one stage of the infectious cycle (Olsen *et al.*, 2012). Motility is considered a virulence factor as it allows strains to interact and adhere to the intestinal epithelial cells (Olsen *et al.*, 2013; Mistry, 2012). Although virulence via motility is important, studies have highlighted non motile strains can cause outbreaks; a non motile isolate of *Salmonella* Typhimurium caused an outbreak of food poisoning in France; investigation showed it was linked to a farm, which produced more than 32,000,000 eggs per year (Le Hello *et al.*, 2012)

All the isolates in the panel produced hydrogen sulphide except for *S.*Senftenberg 775W and this is in line with data published previously working with this isolate (Henry *et al.*, 1969; Yi *et al.*, 2014). Within the limitations of the API 20E which only looks at biochemical reactions involved with twenty assays, no clear discrimination was observed in the profiles of factory, clinical and veterinary isolates, suggesting a similar metabolic strategy is adopted by all the strains. The API Microsystems are a trusted established format for the identification of microorganisms at the species level. Phenotypic systems are classed as the `gold standard' when identifying bacteria in clinical laboratories. Current literature reveals mixed reviews about the use of API kits for the identification of microorganisms; some studies have used API screening for the identification of *Salmonella* and reported a high sensitivity and specificity for the test in comparison to other methods (O'Hara *et al.*, 1992; Peele *et al.*, 1997; Nucera *et al.*, 2006), however other studies have criticised the accuracy of the API20E test in comparison to more advanced systems such as the Vitek (Aldridge *et al.*, 1978; Robinson *et al.*, 1995). Although the API20E has limitations in the scope of the reactions it uses, the benefits are it is a rapid, repeatable pre-screen that can identify major biochemical differences across bacterial strains. As the API20E only uses 20 biochemical reactions, for a more in depth investigation of biochemical differences Biolog Microarrays are utilised as they employ 94 biochemical tests.

2.7 Conclusion

In conclusion, the isolates tested in the panel were confirmed as *Salmonella* and were all motile, the strains isolated from the factory environment shared common phenotypic characteristics with human, clinical and veterinary strains. The API profiles of *Salmonella* from the three groups were identical and did not demonstrate any differences across the strains, with the exception of *S*.Senftenberg 775W which was negative for production of hydrogen sulphide. Analysis of cell length as revealed by SEM did not highlight any significant differences in the gross morphology of the cells and all the cell lengths were within expected parameters. A more detailed analysis of the metabolism of the *Salmonella* strains was undertaken using a high resolution phenotypic microarray (Biolog Inc. Microbial Identification Systems GEN III) as described in the following chapter.

3 Chapter 3 Comparison of metabolism using a phenotypic microarray

3.1 Introduction

The contamination of food products with *Salmonella* is a leading cause of gastroenteritis worldwide (Iwamoto *et al.*, 2010) and determining the survival characteristics of the organism under stressed conditions is fundamental. There are over 2500 different serotypes of *Salmonella* and *S.*Typhimurium and *S.*Enteritidis are the serotypes most commonly isolated from patients presenting with disease symptoms, however reports show an emergence in outbreaks associated with food factories caused by less frequently isolated serotypes including *S.*Montevideo, *S.*Schwarzengrund and *S.*Senftenberg (Rushdy *et al.*, 1998; Behravesh *et al.*, 2010; Gieraltowski *et al.*, 2013). Therefore, characterising the metabolic characteristics of these food factory isolates using phenotypic microarrays isolates and comparing metabolism with *Salmonella* isolated from veterinary and clinical settings may provide useful information to differentiate the food factory isolates.

The recognisable characteristics of a cell are referred to as the phenotype; in this context the phenotype of a cell was determined using a phenotypic microarray. There is a broad application of the phrase `phenotype' to other types of analysis including analysis at the genomic level; however the analysis in this context was geared towards an organism's ability to grow and its profile of metabolic requirements.

Like many other organisms, *Salmonella* are able to grow and survive in a range of environmental niches; this is dependent on two fundamental aspects; the biotic and abiotic factors. Firstly all bacteria must be able to utilise the basic elemental nutrients available in the environment that are essential for growth, these are called the biotic factors and include; Carbon, Nitrogen, Phosphorus, Sulphur, Oxygen and Hydrogen. Secondly, bacteria must be able to overcome environmental pressures which may inhibit growth, these are recognised as the abiotic factors and consist of; temperature, nutrient deprivation and toxic chemicals. Therefore, exploring the growth phenotypes of an organism aids in understanding the fundamental aspects of cellular genome and the evolution of organisms (Bochner, 2009). Previously, in the process of metabolic phenotyping individual reactions were measured in test tubes; the introduction of the API system allowed the simultaneous measurement of up to 50 tests at one time. However, performing tests on individual metabolic pathways is time consuming and it is important to look at networks or associated pathways that may be co-

expressed or co- inhibited. Therefore looking at arrays of metabolic reactions rather than individual reactions is more representative of the types global regulation that bacteria employ, thus, simultaneous determination of multiple metabolic pathways is crucial.

3.2 The use of the Biolog system for simultaneous determination of metabolic characteristics

The Biolog system is based on a 94-well plate format; it permits the simultaneous determination of a range of metabolic characteristics. It incorporates an array of biochemical tests to determine and quantify the utilisation of amino acids, carboxylic acids, esters, salts, fatty acids, hexose acid, hexose phosphates and reducing agents. Bacterial cells are incubated in a defined culture medium with a tetrazolium redox dye. During the incubation period there is increased respiration in the wells where cells can utilize the particular carbon source or ingredient in the culture medium which is recorded as a purple colouration in the well (Biolog, 2008).

Measuring respiration instead of growth has several advantages; most importantly it is a more sensitive measurement as cells are more inclined to respond metabolically by respiring rather than growing. It is also useful for the detection of those cellular pathways for example the formate dehydrogenase pathway which is only detected via respiration and not growth. This pathway is well documented for enteric pathogens including *Salmonella* (Bochner, 2009).

Bacterial strains can be differentiated depending on the carbohydrates they utilise and the acids they produce (the Biolog plate contains a range of carbohydrates including lactose, glucose, sucrose, fructose to name but a few). It is based on heterotrophic metabolism for ATP production; which is pivotal for biosynthesis, maintenance and reproduction. Carbohydrate utilization is useful in the investigation of differences in bacterial strains as all bacteria have a unique collection of enzymes which oxidise energy sources (Parkin *et al.*, 2012).

The Biolog assay works on the basis that following oxidation, by-products are generated and pH indicators are added to the biochemicals to detect the amount of metabolic acids produced. Whereas in the API 20 E test strips, some reactions must have reagents manually added following incubation, for example the indole test requires a drop of James reagent and the TDA test has a drop of TDA reagent added following incubation. These reagents then react

with the bi-products or metabolic pathway intermediates to provide an indication of whether the energy source has been used and this is visualised by a colour change (Johnson and Schwarz, 1944; Mistry, 2012).

Evaluating any potential trends in the profiles generated from micro-array investigations can be challenging to determine via simple observations because of the volume and intricacy of the data produced. Principal component analysis (PCA) is a popular exploratory data-propelled method that is available for the analysis and representation of this volume of data, principle components analysis is valuable in revealing similarities and differences in metabolic profiles that may be concealed within complex data sets. It is also a powerful tool for analysing patterns in high dimension data as once trends have been identified, the data can be compressed by reducing the number of dimensions, leaving only the most influential factors. The observations can then be displayed on a map where each data point is denoted an eigenvalue and an eigenvector and this revealS patterns of similarities across strains; whereby strains that are metabolically similar cluster together (Baumgartner *et al.*, 2000; Abdi and Williams, 2010; Jolliffe, 2014).

Organisms evolve a metabolic profile which is more closely mapped to the environment in which they exist, they express genes required if certain reagents or substrates are available in order to make optimal use of their metabolic system (Gibson, 2008; Seshasayee *et al.*, 2009). Therefore, it can be assumed that the metabolic array we are generating is likely to be influenced by the environment the *Salmonella* strains are isolated from if the environment is a strong driving factor.

The aim of work described in this chapter was to use the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ to produce detailed profiles of *Salmonella* isolated from factory, clinical and veterinary isolates for further exploratory analysis using PCA. This approach would reveal differences in metabolic profiles between the *Salmonella* isolates which may be indicative of metabolic adaptation to particular environments.

3.3 Materials and Methods

3.3.1 Phenotypic microarrays using the Biolog Microbial ID plate

All strains in the challenge panel were grown on Nutrient agar and incubated at 37°C for 24 hours. The Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ and Inoculating Fluid A (IFA) (Technopath, Ireland) were removed from 4°C storage and allowed to reach room temperature before use. Following incubation of the Nutrient agar plates, a single colony was used to inoculate the prepared IFA to produce a homologous emulsion, according to manufacturer's instructions. A volume of 100μL of the IFA suspension was dispensed into each of the 96 wells in the Microbial ID plate; the plates were covered with their lids and incubated at 37°C for 24 hours. The negative wells remain colourless, as does the negative control well (A-1) with no carbon source. The positive control well (A-10) was used as a reference for the chemical sensitivity assays in columns 10-12. Following incubation the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ were analysed for end-point data by reading the absorbance of each well, containing redox dye tetrazolium, at 600nm in a Biotek plate spectrophotometer (Biotek Synergy HT, UK) coupled to a PC running Gen5 data analysis software (Biotek, UK).

Table 5 the selection of the assays in each of the 94 wells in the Biolog Inc. Microbial Identification Systems GEN III MicroPlate

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butryric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Table 5: The GEN III MicroPlate™ test panel utilises the phenotypic pattern obtained from the biochemical tests, to identify and profile a range of both gram positive and negative bacteria, the plate consists of 71 carbon source utilization assays and 23 chemical sensitivity assays which make up the biochemical tests. The nutrients and biochemicals are prefilled and dried into the wells of the MicroPlate. Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon sources or the resistance to inhibitory chemicals

3.4 Data preparation and normalisation

The absorbance data from the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ data was exported from the plate reader software to a Microsoft Excel spreadsheet. These data were then ordered whereby the serotype of each isolate was added in a column and the affiliated 96 absorbance values for each isolate were added in adjacent rows. In a separate sheet the carbon utilization data were normalised by subtracting the negative control from the corresponding test wells. The chemical sensitivity variables were normalised in a similar way in that each of the wells was divided by the positive control value.

3.5 Global visualisation of data

Line graphs were produced to visualise global trends in the utilization of metabolites by the different strains. The numerical raw and adjusted data were then exported into Statistica (version 10, USA) whereby a range of analyses was conducted. Firstly, descriptive statistic tables were firstly produced to show a range of summary statistics including the mean, mode and quartile range values for each well. Explanatory data analysis was applied to summarise the large volume of data produced in the descriptive tables through the production of box and whisker plots, which allowed the visualisation of the shape of the distribution, its central value, and its variability. Lastly, multivariate analysis of the data was performed using the Principal Components Analysis tool. The biochemical test values formed the basis of any potential metabolic 3differences amongst factory, clinical and veterinary isolates of *Salmonella.*

Analysis was conducted on both the raw and normalised data and demonstrated that no changes in the grouping occurred as a consequence of normalising the data. Table 10.1 in the appendix shows descriptive statistics for absorbance values in each well, the mean, mode, median, upper& lower quartiles ranges are all highlighted. Some wells showed large variability in the range of absorbance values in comparison to others.

Exploratory analysis of the normalised data

Figure 25 Box plot to show the overall distribution of the strains based on carbon utilization

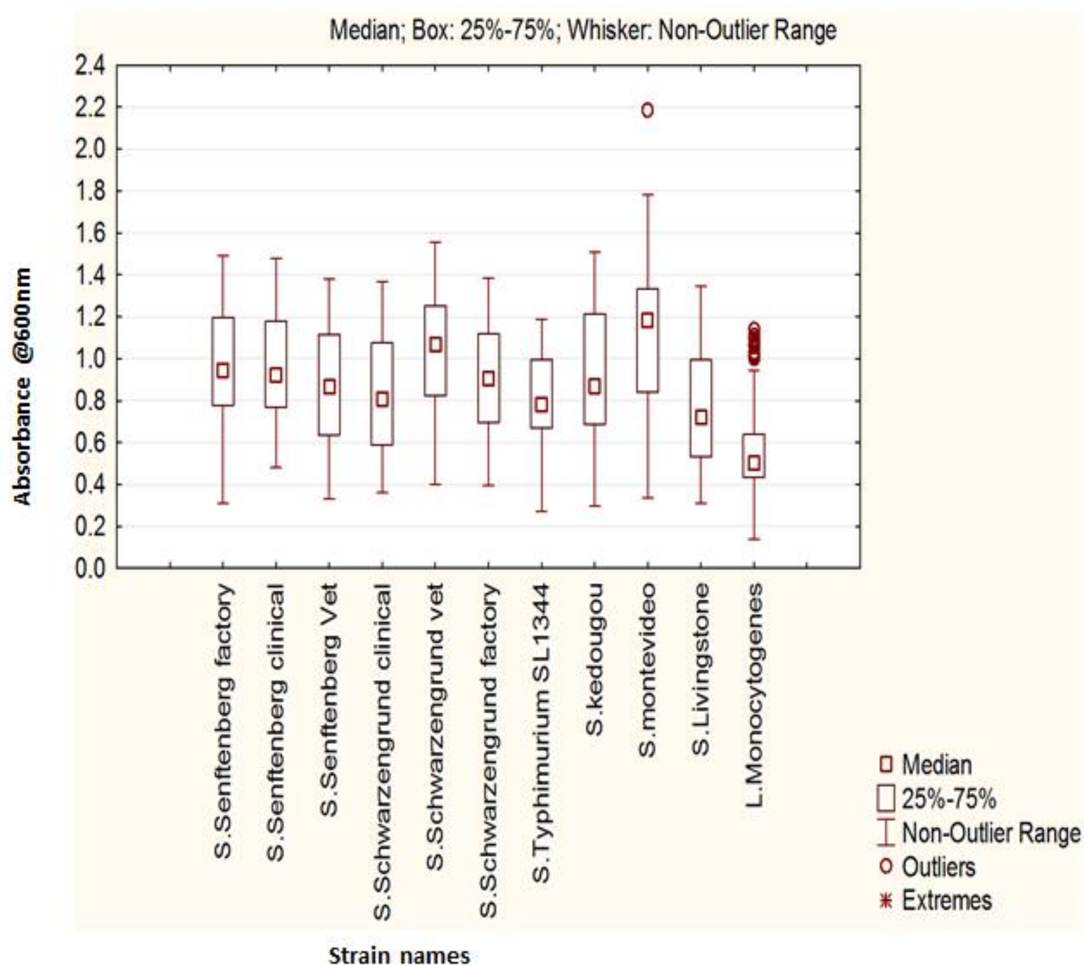


Figure 25: shows a summary box and whisker plot revealing the overall distribution of the strains based on the metabolism of the seventy one carbon utilization wells. Most of the isolates show a reasonably symmetrical distribution with relatively few extremes, with the exception of *S.* Montevideo and *L.monocytogenes.*

Figure 26 Summary Box and Whisker plot of all the biochemical tests of the normalised data

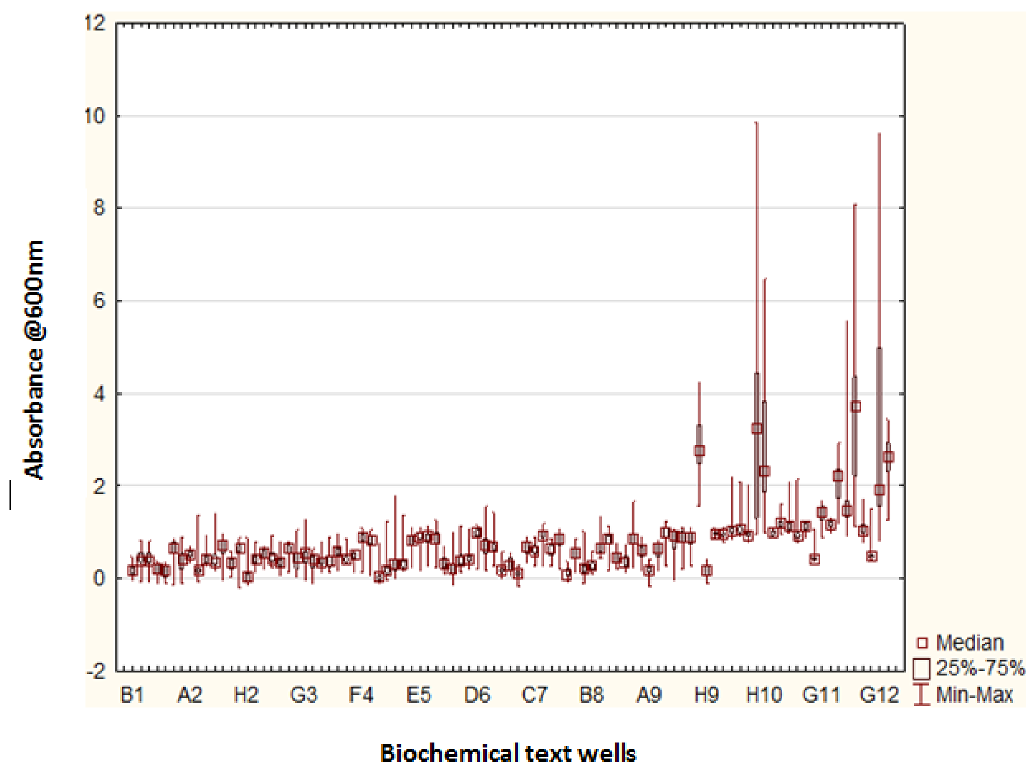
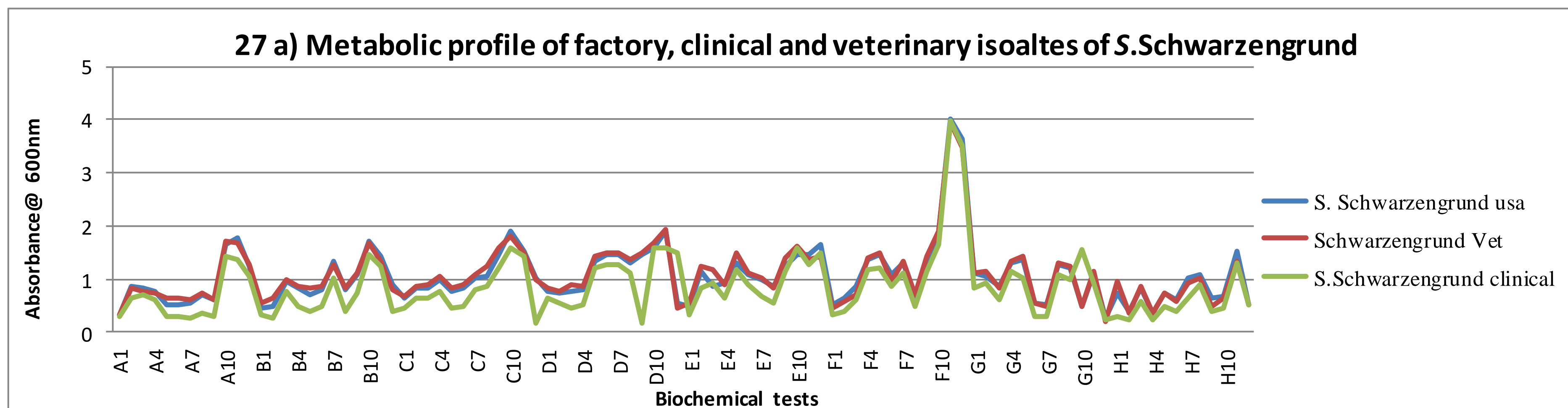


Figure 26: the summary box and whisker plot shows the distribution of the normalised variables. Most of the carbon utilization assays show a reasonably symmetrical distribution with relatively few extremes. Due to the scale it is difficult to identify all the biochemical tests on the X axis, however some of the chemical sensitivity wells (B11 to H12), shown on the right of the plot show a larger cluster of extreme values.



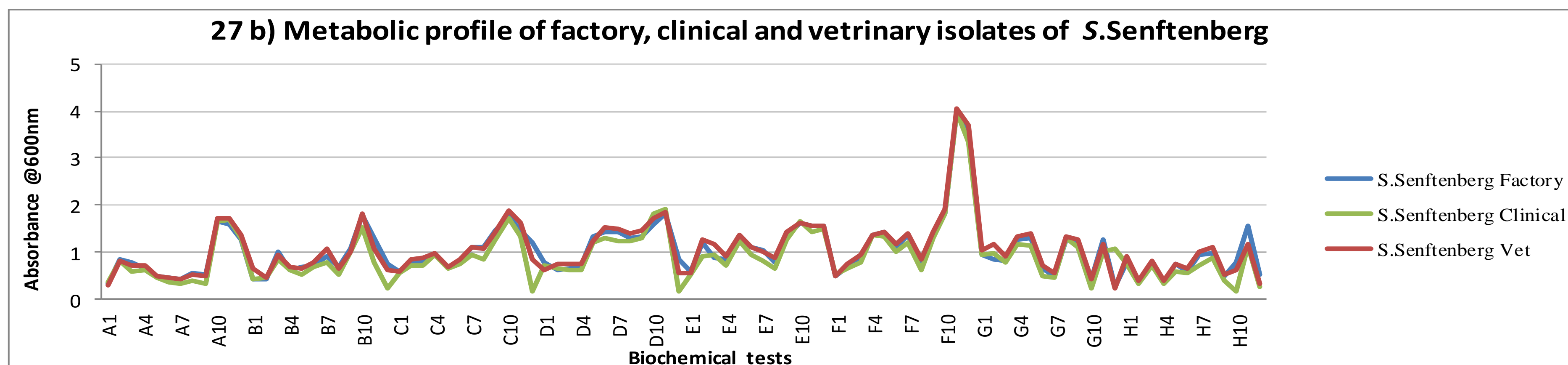


Figure 27 a & b show an overview of the metabolic profile of the two serotype matched strains in the panel.

There are no obvious metabolic differences that can be observed amongst the isolates and the same profile exists between the two serotypes with notable peaks at A10, B10, C10 and F10, all being consistent across the two strains.

3.6 Microbial Identification Systems GEN III MicroPlate assays.

All of the *Salmonella* isolates were investigated for their ability to metabolise a number of different carbon sources including carboxylic acids, esters, fatty acids, hexose acids, amino acids, hexose phosphates, sugars, reducing power reagents and salts. The absorbance readings at 600 nm were recorded after 24 hour incubation of the isolates with each biochemical test, the results showed readings ranged from zero to four. Visualisation of the data using the line graphs reveals a broad similarity between the metabolic profiles of the isolates where as some differences in utilization of certain metabolites can be seen visually*, L.monocytogenes* has a distinctively different pattern of metabolism from the *Salmonella* isolates, whereby it underutilizes many reagents including sorbitol, gelatin, pectin, gluconic acid and arginine. There is also notable elevated utilization for the factory isolate of *S*.Montevideo in its utilization of some reagents including glucose, sorbitol, galactose and fructose to name but a few. The complexity of the data makes it difficult to accurately identify changes in the global metabolic profile; therefore principal components analysis was applied to the data to assist in revealing any significant patterns embedded in the profiles of the factory, clinical and veterinary isolates.

3.7 Principles Components Analysis

Figure 28 a plot of the eigen values of the raw and normalised data showing the significance of each of the factors that form the basis of PCA

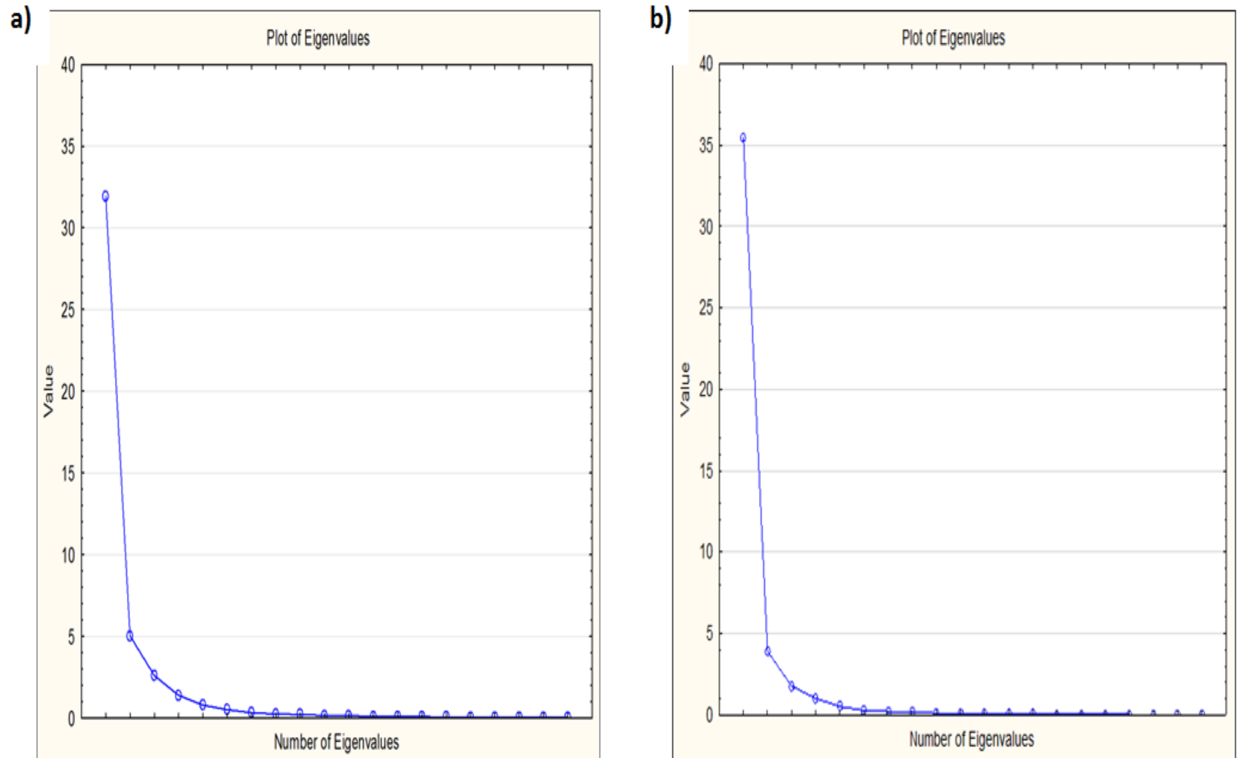


Figure 28 a) shows the eigen values of the raw data b) shows the eigen values of the normalised data. As the name suggests a PCA condenses the multiple factors into principle factors. It works by reducing the number of variables and is based on eigen values and eigen vectors. Eigenvectors and values coexist in pairs. An eigenvector is described as the direction and the eigen value is a number, expressing the variance in a particular direction. Therefore the principal component is the eigenvector with the highest eigenvalue. The plot highlights that normalising the data did not cause a change in the grouping of the data as the plots are almost identical. The highest proportion (80.57%) (factor 1) of the variance is influenced by the first eigen value. 8.88% of the variance is influenced by the second eigen value (factor 2) and only 4.04 % of the variance in the data is influenced by the third eigen value (factor 3).

Figure 29 Scatter graph of factor loadings of factor 1 versus factor 2 as calculated by 2D Principal Components Analysis

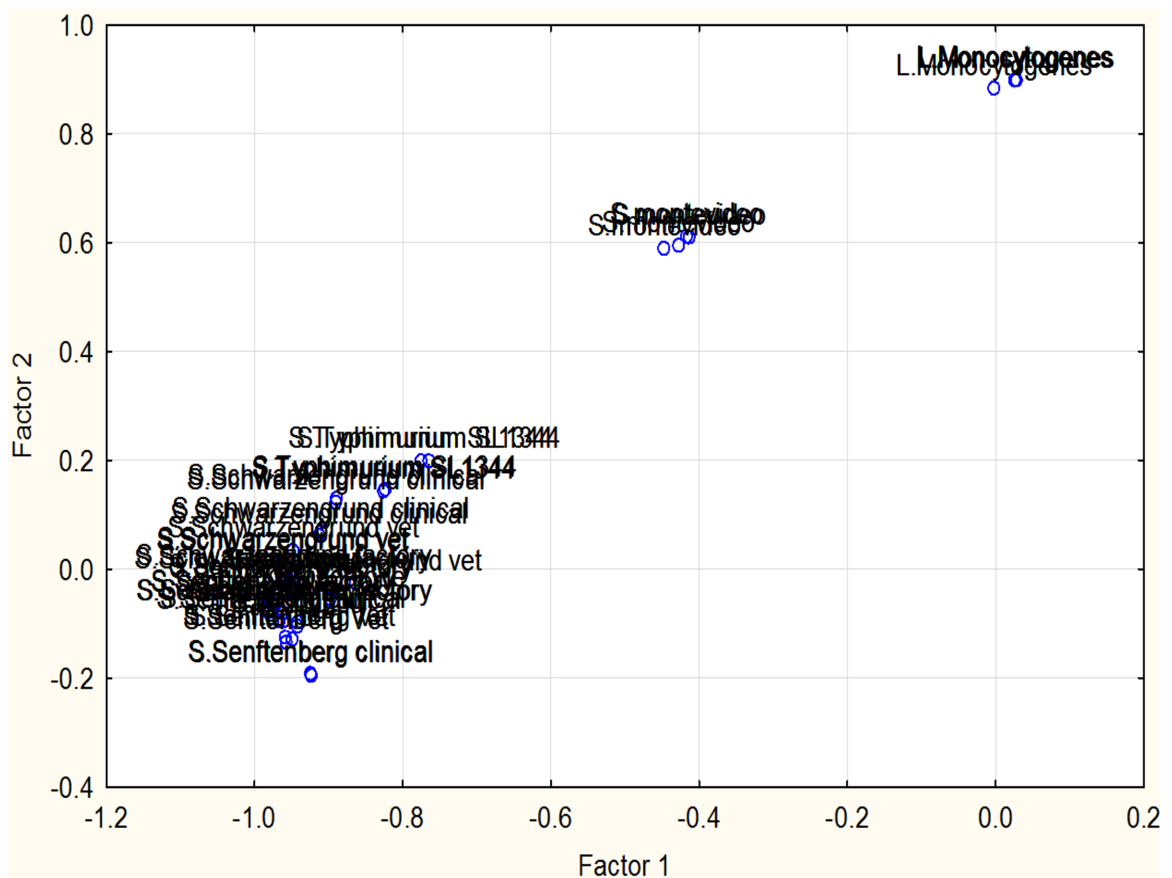


Figure 29: A 2D PCA from the Microbial Identification System GEN III MicroPlate. The output is based on all the absorbance readings of the raw unadjusted data of *L.monocytogenes* 11994 and clinical, factory and human isolates of *Salmonella*, whereby the components are serotypes, factor 1 by factor 2. The *L.monocytogenes* is in a noticeable cluster away from the *Salmonella* isolates as might be expected as it is a different genus. The factory isolate of *S*.Montevideo is also in a distinctive group from the rest of the *Salmonella* isolates. The remainder of the *Salmonella* isolates from the three environments did not form distinctive clusters.

Figure 30 Scatter graph of factor loadings as calculated by 2D Principal Components Analysis for the normalised data

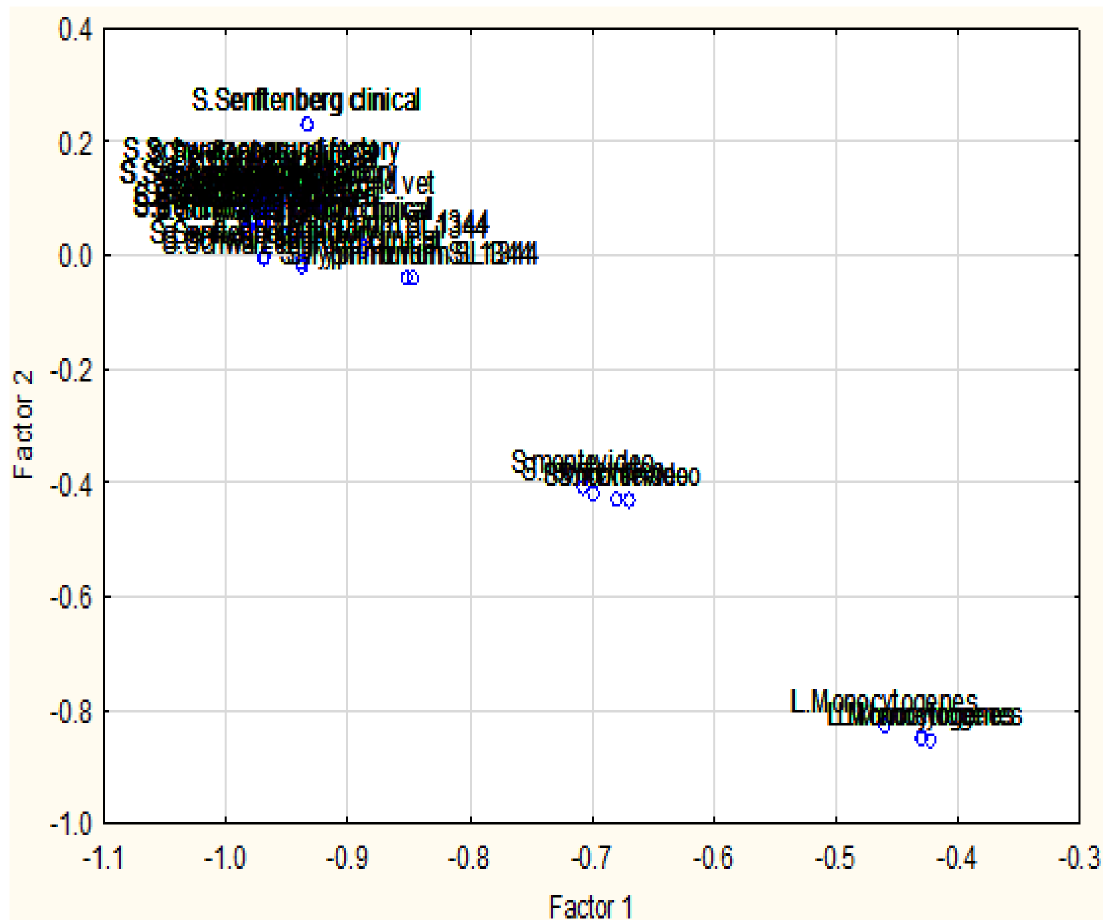


Figure 30: A 2D PCA of the normalised data of *Salmonella and L.monocytogenes* 11994. This highlights that normalising the data has not introduced any differences in the grouping of the data and the plot is almost identical to the 2D PCA of the raw data, with *L.monocytogenes* in a noticeable cluster away from the main cluster of *Salmonella* isolates and the factory isolate of *S*.Montevideo in a distinctive group from the rest of the *Salmonella* isolates.

Figure 31 Scatter graph showing 2D Principal Components Analysis of the *Salmonella* isolates

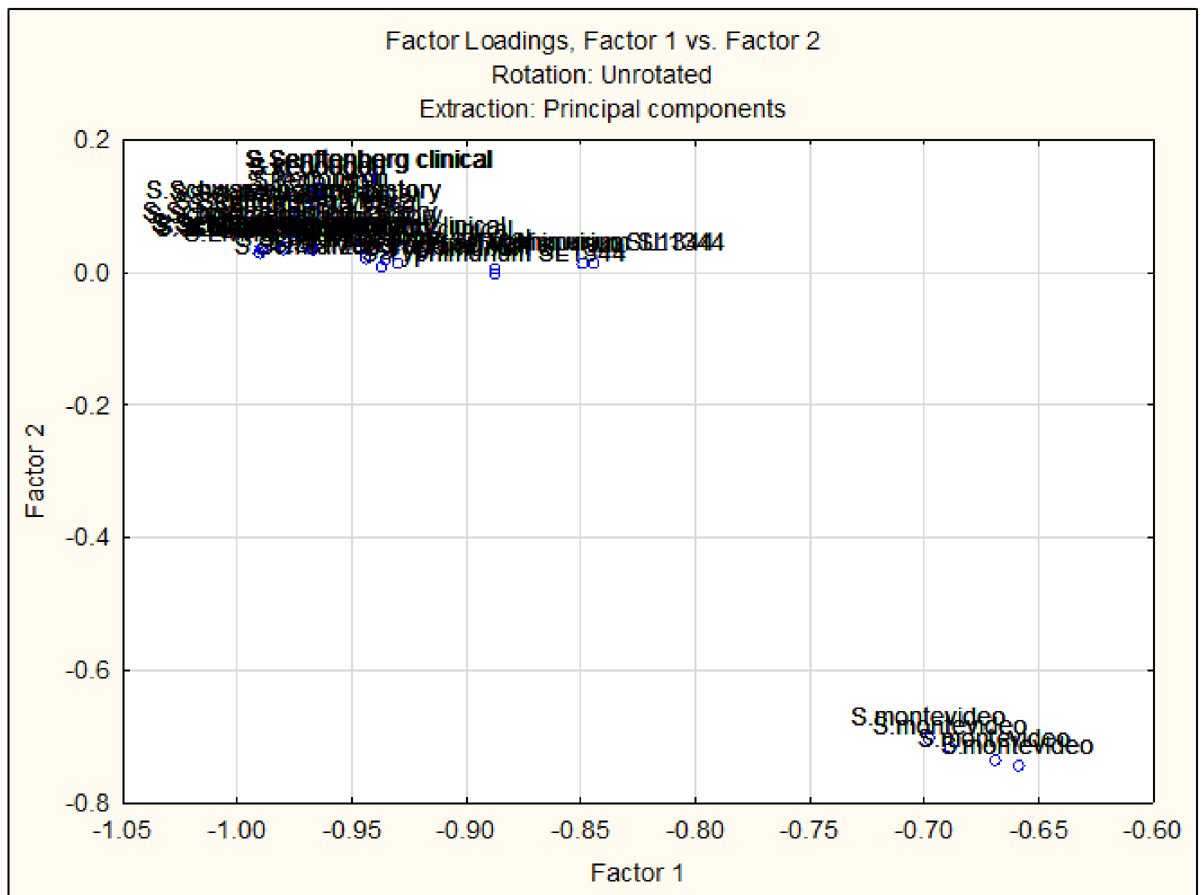


Figure 31: A 2D PCA from the Microbial Identification System GEN III MicroPlate, focussing only on the *Salmonella* isolates. The output is based on all the absorbance readings from the carbon utilization assays of the raw unadjusted data and indicates that there are significant differences in the way *S*.Montevideo utilizes the carbon sources. Whereas results focusing only on the chemical sensitivity assays showed no differences and all *Salmonella* isolates formed an undistinguishable cluster.

Figure 32 A 3D principle components analysis Scatter plot of microbial data

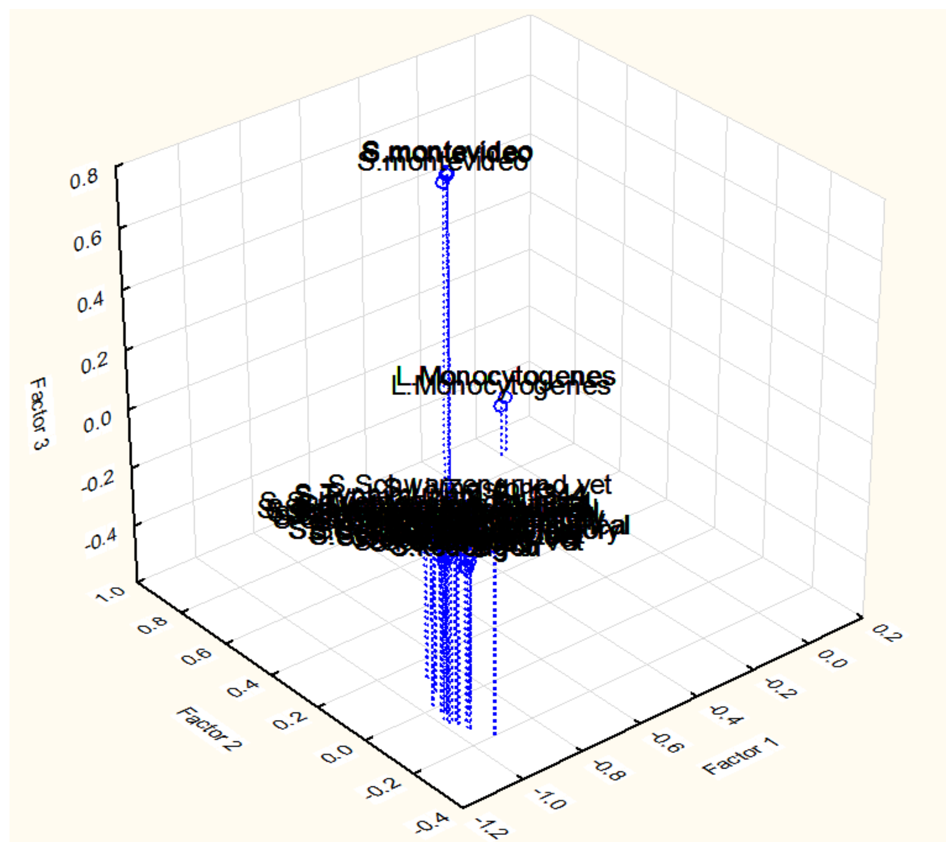


Figure 32: A 3D PCA from the Microbial Identification System GEN III MicroPlate, the output is based on all the absorbance readings of the normalised data of the panel of *Salmonella* isolates and *L.monocytogenes* 11994. With the introduction of a third factor, which provides more opportunity to cluster diverse organisms, the *Salmonella* still cluster together as an undifferentiated group, the *L.monocytogenes* remain together and distinct and the *S*.Montevideo forms a distinct cluster.

To understand further the driving factor behind this distinctive clustering for the factory isolate of *S*.Montevideo, a table of the average absorbance values for each of the biochemical tests was assembled, this facilitated the visualisation of the key differences in the isolates metabolism and the data are displayed in Table 6.

Table 6 Biochemical tests driving the distinct clustering of *S.*Montevideo in the PCA

Strain	a-D-Glucose	D-Sorbitol	D-Mannose	D-Mannitol	D-Maltose	D-Frucose	D-Trehalose	D-Galactose	L-Rhamnose	b-Methyl-D Glucoside
*S.*Senftenberg factory	0.41525	0.41525	0.46925	0.374	0.56525	0.61025	0.39725	0.72075	0.86525	0.4405
*S.*Senftenberg clinical	0.2785	0.2785	0.32475	0.267	0.2115	0.34225	0.22125	0.516	0.5205	0.2575
*S.*Senftenberg Vet	0.426	0.426	0.44	0.3815	0.4495	0.4675	0.3865	0.65625	0.6925	0.2265
*S.*Schwarzengrund clinical	0.36525	0.36525	0.34375	0.369	0.31975	0.36375	0.31625	0.55825	0.51025	0.25175
*S.*Schwarzengrund vet	0.5885	0.5885	0.52325	0.5445	0.49825	0.45575	0.39325	0.63125	0.74275	0.4335
*S.*Schwarzengrund factory	0.5785	0.5785	0.4175	0.543	0.4055	0.43825	0.3455	0.56025	0.627	0.44575
*S.*Typhimurium SL1344	0.3833175	0.383318	0.274075	0.37675	0.385328	0.38825	0.431483	0.5845	0.56575	0.219425
*S.*Kedougou factory	0.33575	0.33575	0.33275	0.23675	0.30475	0.43725	0.29575	0.49725	0.568	0.365
*S.*Montevideo factory	0.7865	0.7865	0.94075	1.30225	0.72525	1.02575	0.6255	0.97475	1.3315	0.87475
*S.*Livingstone factory	0.45975	0.45975	0.38875	0.33775	0.39125	0.38275	0.259	0.549	0.668	0.296

Table 6 lists some of the discriminatory tests that were over - utilized by the factory isolate of *S.*Montevideo in comparison to the other strains. The values represent the average absorbance for each substrate based on four experiments and Results for *S.*Montevideo are shown in red. The table highlights that *S.*Montevideo is able to ferment Glucose and a majority of the sugar alcohols (Sorbitol, mannose, mannitol, maltose, and fructose) better than the other isolates in the panel.

Figure 33 histograms representing selected sugar metabolism

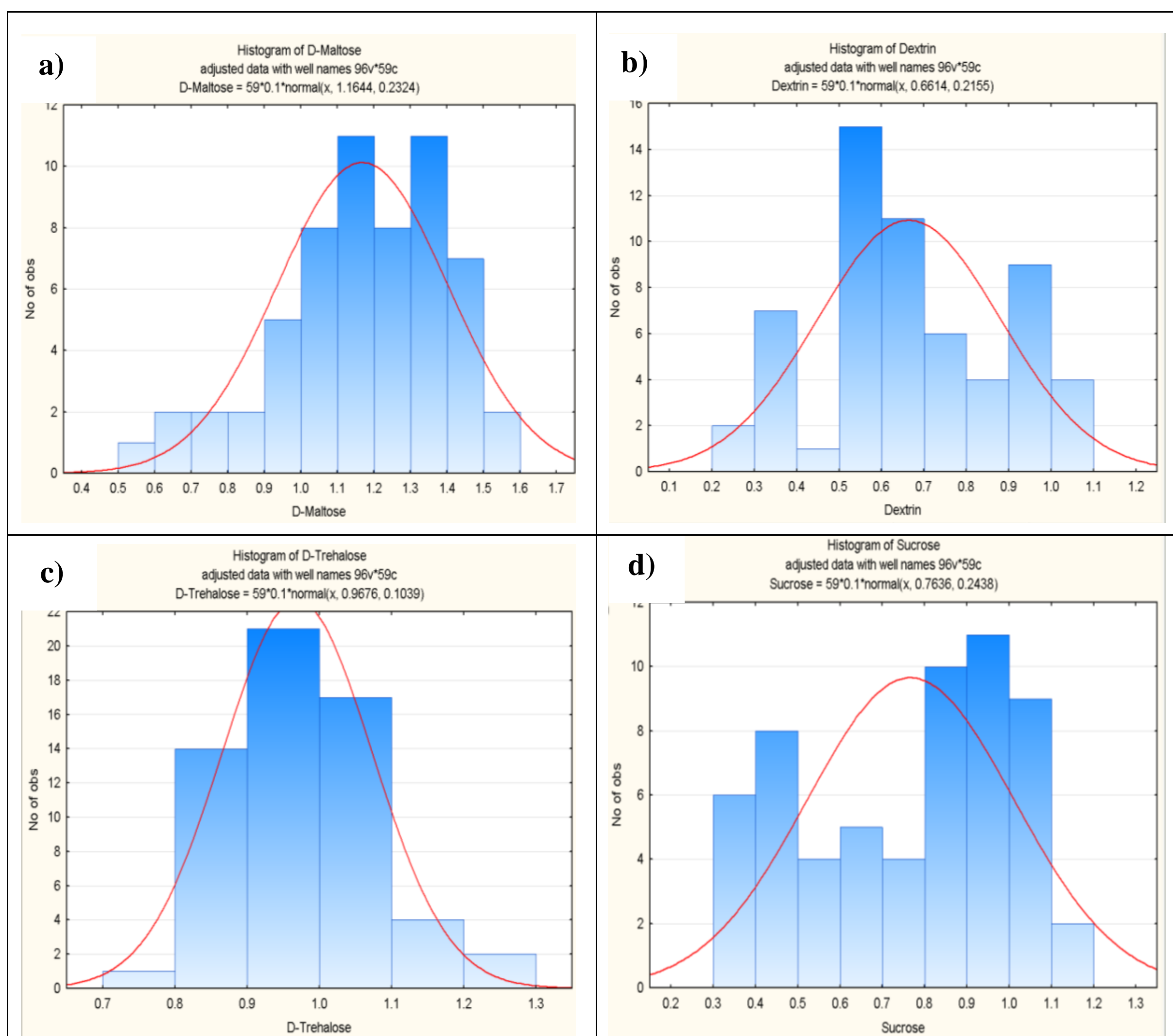


Figure 33 a-d: Frequency histograms representing selected sugar metabolism, each histogram represents the most frequently observed absorbance readings within the population. The X axis represents the absorbance values and the Y axis represents the frequency of observations. The graphs exhibit varying degrees of fit to a normal distribution (the curves fitted to each histogram). For example, for D-Trehalose, the most frequent absorbance that was demonstrated in 21 of the wells was between 0.9 and 1.0, whereas there was only one observation of an isolate that fermented Trehalose with its lower capacity of 0.7 and there were only 2 isolates that fermented it at its higher capacity of 1.3. Overall, the histograms reveal some curves were a good fit, some were skewed, and some more asymmetrical which is as expected as each strain utilizes the biochemical reagents differently.

3.8 Discussion

The aim of this chapter was to metabolically profile a panel of factory, human clinical and canine isolates of *Salmonella* and *Listeria monocytogenes* using GEN III Biolog Microbial Identification plates. Profiling microbial physiology is a useful tool for defining a pathogens metabolism during growth and survival, as it allows the description of cellular functions involved in bacterial stress such as withstanding pH, nutrient starvation and utilization of carbon sources all of which may limit growth and respiration. It also permits the identification of any potential differences in metabolism between isolates (Bochner, 2009). Comparison of phenotypic characteristics and bacterial response to stress may provide an indication of potential adaptation to the food factory environment.

The Phenotypic microarray plates are an ideal format for the simultaneous analysis of multiple phenotypic differences across a panel of isolates. The presence or absence of the purple reporter dye is a reproducible, sensitive and a quantitative measure (Tracy *et al.*, 2002). However the complexity of the data obtained given the number of wells being observed can be difficult to interpret therefore can benefit from exploratory analysis. The Principal Component Analysis (PCA) of the Biolog data indicated that the majority of the *Salmonella* isolates shared similar metabolic capabilities but there were two distinct isolates which did not cluster with the main group. These two isolates were the factory strain of *S.*Montevideo and the control organism, *L.monocytogenes*. The biochemical reactions driving the differences between the major group of strains and outlying factory strain of *S.*Montevideo were primarily Glucose and the sugar alcohols D- Sorbitol, D- Ramnose, D-Mannose, D-Mannitol, D-Fructose, D-Maltose, D-Trehalose, D-Galactose and b-Methyl-D Glucoside. The outliers exhibited an enhanced rate of metabolism within the 24 hour test period in comparison to the rate observed for the other isolates.

The sugar alcohol group plays a key role in glycolysis and glycolysis is a crucial process for both aerobic and anaerobic respiration and occurs in most organisms (Kim and Surette, 2004). In brief, *Salmonella* uses glucose as a form of nutrients in a series of catabolic reactions that convert sugars (maltose, galactose, D-fructose D-mannose) into pyruvate and the results in the simultaneous synthesis of ATP and NADH. Several carbohydrates catabolized by glycolysis are imported through the phosphotransferase (PTS) system; this moves phosphate from the glycolytic intermediate phosphoenolpyruvate to a cascade of enzymes, consequently triggering the phosphorylation of the transported sugar. In summary, as Figure 35 shows, Enzyme 1 (E1)

transfers a phosphate group from phosphoenolpyruvate to Enzyme 2 (EII) by the means of HPr, the transportation and phosphorylation of the incoming sugar is dependent on enzyme 2 (Fraenkel *et al.*, 1963; Bowden *et al.*, 2009; Schaechter, 2009). This process releases energy required for growth and reproduction, and the utilization of glucose and sugars inevitably results in enhanced growth and respiration in the organism. An increase in β-methyl-D glucoside was also observed; typically this is found in plant material and most enteric pathogens can use it as a carbon source by cleaving β-glucosides to produce glucose-6-P this can then be integrated into glycolysis (Faure, 2002; McCarroll., 2008)

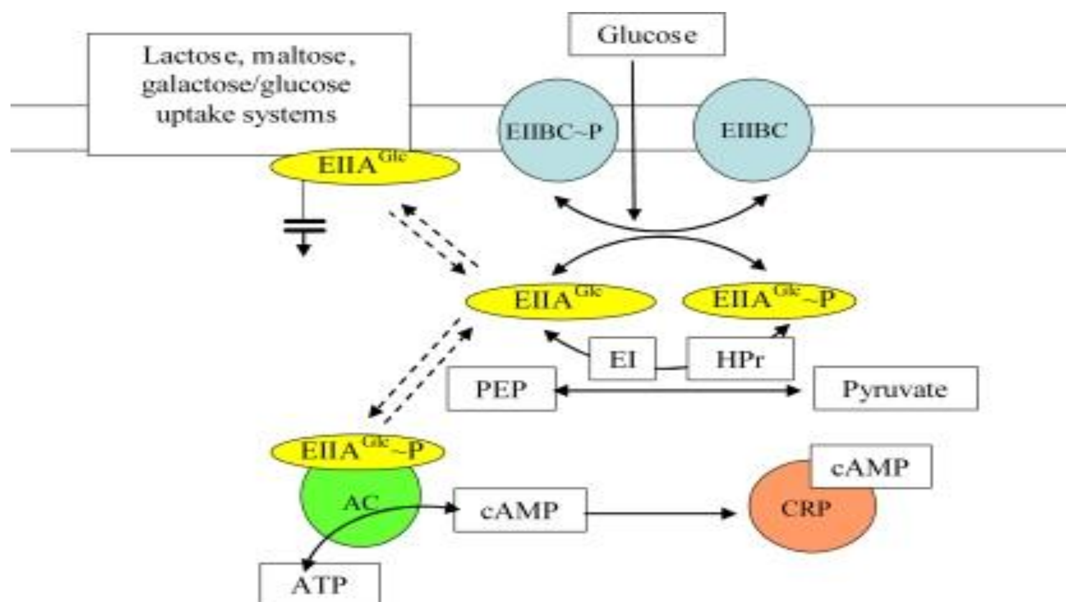


Figure 34 an overview of the glycolytic pathway which converts hexose sugars into ATP and pyruvate

The results also reveal a high absorbance for Trehalose in *S.*Montevideo in comparison to other *Salmonella* isolates. This is an important disaccharide that derives from UDP-glucose and glucose-6-phosphate and is said to be implicated in protecting cells from injury caused by high osmolarity, heat, oxidation, desiccation and freezing (Reina-Bueno *et al.*, 2012). Cánovas *et al.* (2001), investigated the mechanism by which high osmolality enhances the thermotolerance of *S.*Typhimurium and showed that Trehalose accumulation is thermoregulated and when Trehalose synthesis is blocked by mutations, results show a growth defect at high temperature in media of high osmolality. They concluded that Trehalose is important for growth at high temperature either for turgor maintenance or for protein stabilization (Cánovas *et al.*, 2001).

Table 7a reveals that D-Glacatose was also utilised better in *S*.Montevideo, other than glucose, several other monosaccharides can also be utilised as carbon sources by *Salmonella*.
D-Galactose is one of the key constituents of the outer core of *Salmonella* Lipopolysaccharide (LPS). The LPS forms an important part of the outer membrane of most Gram negative pathogens as it primarily is responsible for the interaction of the cell with the external environment and therefore playing a major role in virulence. Lipopolysaccharide generally comprises three structural sections: lipid A, core oligosaccharide (outer and inner) and the O-polysaccharide side chain (O-PS) (Brooks *et al.*, 2008). The factory strain of *S*.Montevideo revealed a higher rate of growth in the environment abundant in D- Galactose which could become a competitive advantage in an environment containing this monosaccharide. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is an online database that provides useful information about high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from genomic and molecular-level information. It highlights the interactions between genomic and chemical reactions thereby acting as computer representation of the biological system (Kanehisa Laboratories, 2010). The database indicates that D-galactose is necessary for vital pathways such as; bacteria chemotaxis, amino acid and nucleotide sugar metabolism, carbohydrate digestion and absorption as well as mineral absorption (Mistry, 2012; Kanehisa Laboratories, 2010).

3.8.1 L.monocytogenes

Listeria monocytogenes is also an important opportunistic food-borne pathogen that can lead to Listeriosis. The capability of bacterial metabolism to adapt to the host cell is an important part of the replication cycle in all intracellular pathogens (Gillmaier *et al.*, 2012). In order to understand the ability of the organism to survive in both extracellular and intracellular environments it is essential to understand more about the ability to metabolise nutrients (Mitchell *et al.*, 1993).

As *Listeria monocytogenes* is a typically Gram positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria, there are inevitably notable differences in its metabolism in comparison to the *Salmonella* isolates (Liu, 2006). Absorbance data reveals that *Listeria Monocytogenes* upregulated several carbon sources including; D-Salicin, D-Cellobiose, D-Fructose, Gentiobiose, N-Acetyl-D Glucosamine and it also showed a higher resistance towards Tetrazolium violet.

There is evidence to suggest that like *Salmonella*, *Listeria monocytogenes* is able to utilize carbohydrates other than glucose for growth (Pine *et al.*, 1989; Premaratne *et al.*, 1991). However glucose in addition to other PTS-sugars like fructose, mannose and cellobiose are the preferred carbon sources for *L. monocytogenes* when growing in defined liquid minimal media (Joseph and Goebel, 2007). Studies have shown that it possesses a phosphotransferase system as well as a cyclic HPr seryl modification system which is a recognised system in the regulation of carbohydrate metabolism in Gram-positive bacteria (Mitchell *et al.*, 1993; Joseph and Goebel, 2007). One study investigating the attachment of *Listeria monocytogenes* cells to stainless steel discs at 21°C, found that replacing glucose in the in D10 media with other carbohydrates including with mannose, cellobiose, fructose and trehalose did not affect cell attachment (Kim and Frank, 1994). Other studies have shown that in the presence of glucose, the metabolism of sugars (including cellobiose, salicin and mannose) was supressed, however when glucose was consumed these sugars were then fermented, this signifies catabolite repression by glucose (Gilbreth *et al.*, 2004).

L.Monocytogenes also significantly underutilised some carbon sources in comparison to the *Salmonella isolates* including; Pectin, a-Hydroxy-Butyric Acid and D-Gluconic Acid. In line with this, a recent study investigated the attachment of *Salmonella* and *Listeria monocytogenes* to bacterial cellulose derived plant cell walls, including pectin which is derived from plant material. The results from this study also suggest that the *Salmonella* strains tested fermented pectin better than the *Listeria* strains. This is valuable as it highlights the potential of pectin to act as a carbon source for key foodborne bacteria in plant derived foods (Tan *et al.*, 2013). Gluconic acid is naturally found in fruit and honey. El-Shenawy & Marth (1990) studied the behaviour of *Listeria monocytogenes* in the presence of gluconic acid and reported that milk containing gluconic acid caused partial to complete inactivation of *L. monocytogenes* (El-Shenawy and Marth, 1990). The survival of *Listeria* spp. under cold conditions is associated with the metabolism of Hydroxy-Butyric Acid, is used as a carbon source by both gram positive and negative cells under stressful (low temperature), nutrient limited conditions (Buzoleva and Chumak, 2000). However in the current study *L,Monocytogenes* under- utilised this carbon source suggesting that it is less capable of storing and metabolising the substance than the *Salmonella* isolates in these conditions.

3.9 Conclusion

In conclusion, the current results indicate that a majority of *Salmonella* strains isolated from the food factory environment shared a common metabolic capacity and were able to use a similar diversity of organic nutrients to the human clinical strains and veterinary isolates. Absorbance data in combination with PCA analysis has allowed for the discrimination of firstly the control organism *L.monocytogenes* on the basis of its distinct metabolic profile; this was expected as *L.monocytogenes* is a different genus and species and has an established different metabolic profile. As well as highlighting the differences in metabolism of the factory isolate of *S.*Montevideo, irrespective of the environment it was isolated from, it was distinct from other *Salmonella* on the basis of a differential metabolic ability to utilise glucose and the sugar alcohol group better than the other *Salmonella* isolates. This type of analysis allows for exploratory data mining of complex data to reveal patterns within those data that might be discriminatory or characteristic of underlying features such as an organism's ability to utilise a different carbon source or have a different resistance profile. On the basis of this chapter the distinctive clustering of the remainder of the *Salmonella* isolates, including those that were serotype matched shows that the isolates are metabolically indistinguishable therefore the ability to utilize carbon sources and differences in chemical sensitivity profiles are unlikely to be contributing to *Salmonella* persistence in the food factory environment.

4 Chapter 4 Modelling growth and survival of *Salmonella*

4.1 Introduction

Modelling the growth and survival characteristics of microorganisms reveals interesting patterns that aid in the control of microbial contamination. The retention of bacteria on food contact surfaces poses a major concern to the food industry, especially in areas where cleaning is difficult, as cross-contamination of products at the manufacturing stage can easily lead to foodborne illness and a product backlash from the public. Although these microbes can be inactivated via adequate thermal processing, studies have indicated that organisms such as *Salmonella*, *Campylobacter* and *L.monocytogenes,* which are commonly associated with the food manufacturing environment, can survive on surfaces from hours to days following initial contact (Bremer *et al.*, 2001b; Kusumaningrum *et al.*, 2003; Wilks *et al.*, 2006; Habimana *et al.*, 2010b).

4.1.1 Design of food equipment

Food factories are designed with a view to facilitate hygiene through the regulation of flow through the factory from the arrival of raw material to the finished packaged end products and many precautions are in place to control the spread of pathogens. As part of the design it is important to ensure that easy to clean materials are used for machinery and surfaces, there are no unwanted openings that allow the entry of contaminants and pests, air intakes are suitably placed, and that the dirty zones where raw materials enter are separate from the clean zones where the end product is kept. Procedures are in place to minimise criss-crossing between the zones and separate routes of entry and movement are designated (Ryser and Marth, 2007; Holah and Lelieveld, 2011). Despite these measures, the food factory environment still provides a niche for the survival of these microorganisms; and although machinery and food processing equipment are designed with a view to prevent contamination (Holah and Lelieveld, 2011; Baker *et al.*, 2012), they still offer an opportunity for microbes to contaminate areas of the equipment and provide a source of cross-contamination. With age and poor maintenance, walls with cracks, damaged machinery, water pipes and

gutters all pose a risk of cross contamination due to the accumulation of microorganisms (Oliveira *et al.*, 2007).

4.1.2 Attachment and survival on food manufacturing surfaces

Various studies have been conducted previously on surfaces typically found in food processing plants, such as stainless steel, granite and plastic (Habimana *et al.*, 2010b; Veluz *et al.*, 2012). Stainless steel is commonly used in factories for working surfaces, pipes, tanks and machinery as it has many properties which make it an ideal surface. For example it is resistant to corrosion and is protected by a layer of naturally occurring chromium oxide on the surface, which is formed when chromium and air combine. Stainless steel is also inert and does not contribute any taint to food products contacting the equipment and it can withstand low and high temperatures, most importantly it is also relatively easy to clean (Euro-inox, 2006). Importantly, stainless steel shows resistance to chemicals used for sanitizing work surfaces such as hypochlorite and peracetic acid (Oliveira *et al.*, 2007; Habimana *et al.*, 2010b).

Temperature and humidity levels fluctuate in food manufacturing environments with the differing levels of activity from processing zones to food packaging. In an attempt to model dry and humid conditions that may be found in food factory setting, Habimana *et al.* (2010b), investigated the survival of *Salmonella* on stainless steel coupons at 12°C with high humidity (85%) and 30°C and low RH (35%). Results showed a rapid decrease in *Salmonella* survival with a low temperature and high humidity combination, this emphasises the importance of controlling these two parameters. Furthermore, a recent study investigating the survival of *Salmonella* strains desiccated on stainless steel and stored at 25 °C with 33% humidity showed that they could survive on stainless steel surfaces for a duration of at least 30 days however survival was not serotype related; the highest and lowest survival was observed for *Salmonella* Typhimurium (Margas *et al.*, 2013).

Other studies have investigated how different serotypes of *Salmonella* adhere to surfaces and evaluated the surface hydrophobicity and surface elemental composition (Oliveira *et al.*,2007). In one study, coupons of steel and polyethylene were immersed in bacterial suspension and their inactivation by biocides commonly used in the food industry was investigated. Results showed that *Salmonella* did attach to both surfaces

and biocides were not effective in inactivating all the microorganisms adhered on both surfaces (Tondo *et al*.,2010).

Dawson *et al.* (2007), investigated the survival and transfer of *Salmonella* Typhimurium from surfaces to food products. Food contact surfaces have the potential to serve as reservoirs of bacteria for extended periods of time. Many factors are involved in bacterial transfer from surfaces to food including; surface type, bacterial contact time with surface as well as food contact time with the contaminated surface. It has been shown that *Salmonella* Typhimurium can survive on surfaces for up to four weeks and cross contaminate food almost immediately (Dawson *et al.*, 2007).

4.1.3 L.monocytogenes as an example of an environmentally persistent organism

L. monocytogenes is also known as a causative agent of foodborne disease, and is often isolated from the food manufacturing environment. Investigations have shown that many cases have arisen due to the contamination of equipment and machinery (Ryser and Marth, 2007; Takahashi *et al.*, 2011). *Listeria monocytogenes* also has the ability to grow and survive in an array of environmental conditions, including low refrigeration temperatures, acidic pH levels and high salt concentrations (Gandhi and Chikindas, 2007). Previous studies have also shown that *L.monocytogenes* on stainless steel coupons soiled with food constituents exhibited a 3 log CFU/coupons survival, considerably higher than that of *Staphylococcus aureus* and *Salmonella* Typhimurium incubated under the same conditions (Takahashi *et al.*, 2011). Similarly, Bremer *et al.* (2001a) highlighted that *L. monocytogenes* inoculated on stainless steel coupons, incubated at 15°C with 75% humidity survived up to 40 days, however decimal reduction times were much lower at 4°C and 20°C. This suggests the importance of the interaction of temperature and humidity, it also revealed that the number of sublethally damaged cells from time 0 to 40 days increased to 91%, indicating that solely using agar for recovery of viable cells is not sufficient and more robust detection methods are required when colony forming units can no longer be detected on agar (Bremer *et al.*, 2001b).

4.1.4 *The viable non culturable state in Salmonella and Listeria monocytogenes*

For many years it was assumed that once bacterial cells were no longer culturable on non-selective agar media they were dead, however recent findings have shown that even after losing cultivity, cells were still viable and enclosed the potential to regrow in a state recognised as "viable but non-culturable" (VBNC) (Oliver, 2005). Bacteria can become VBNC due to environmental stress such as temperature, pH and nutrient deprivation, whilst in this state they are considered live but do not grow or divide, both *Salmonella* spp and *Listeria monocytogenes* alongside a host of other enteric pathogens (both Gram -positive and negative) are known to enter this state. The ability to switch to the VBNC state is a grave concern for microbiologists in the food industry especially as cells have the potential to switch to the infectious stage once in the host organism and many laboratories do not routinely test for VBNC (Nascutiu, 2010). Methods employing nucleic acid stains can be used to illustrate when bacterial cells have entered this physiological state. The LIVE/DEAD® BacLight™ Bacterial Viability Kit available from Life Technologies (UK) is a stain used to explore cells entering the VBNC state. It employs two nucleic acid stains—green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO® 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red.

4.1.5 *Growth profiling*

Cell division increases the number of cells within a population and this can be studied by analysing a bacterial growth curve; characteristically four phases of bacterial growth are recognised and documented. Firstly, when a medium is inoculated with bacterial cells, the initial population of cells remains constant, this is described as the lag phase. Whilst there is no noticeable cell division occurring, during this phase other key steps such as the synthesis of enzymes and proteins, increase in mass and metabolic activity may be occurring. The length of the lag phase is dependent on a range of factors such as the initial inoculum, time required for the recovery from shock or physical impairment caused by the transfer into the medium and time necessary for

the synthesis of enzymes required to metabolize the substrates in the medium. The second phase is called the exponential or log phase, in this phase the cells divide via binary fission and grow in a linear manner. It primarily depends on the composition of the medium and growth conditions /stress. Inevitably, growth in this phase is limited by either the depletion of nutrients, space or the build-up of inhibitory metabolites and end products which lead to the third phase, the stationary phase. The final phase is cell death, where cell counts decrease geometrically (Zwietering *et al.*, 1994; Ullmann, 2012; Stumbo, 2013).

4.1.6 The extremes Salmonella encounter in the food manufacturing environment

Growth profiling in nutrient limited media in addition to nutritious media at different temperatures is vital in understanding how microorganisms survive in sub-optimal environments. An enteric pathogen like *Salmonella* is exposed to numerous stressful environments throughout its life cycle and the mechanism by which it reacts to different and multiple stresses are respectively complex. Stress can be defined by a number of variables, including when the bacterial cell encounters dramatic changes in the environment. In the laboratory environment, *Salmonella* may encounter stress naturally through a limited supply of nutrients in addition to *Salmonella* entering the stationary phase of growth (Rychlik and Barrow, 2005). Organisms present in factory environments are prone to experiencing temperature fluctuations and varying levels of nutrients due to the different stages of the manufacturing cycle; from periods of relatively high temperatures in the processing zone, to cooler temperatures in the drying and packaging areas. Therefore, investigating if factory strains of *Salmonella* demonstrate an enhanced growth rate, compared to strains from clinical and veterinary environments, which thereby allows them to rapidly establish themselves in the factory environment niche is important.

It is essential to understand how *Salmonella* is introduced and survives in food processing environments. Previous studies conducted have observed many serotypes of *Salmonella* are capable of adhering to factory surfaces, however little research has been conducted comparing the survival of different isolates of *Salmonella* from the same serotype. In order to eliminate *Salmonella* contamination in food factories, it is important to investigate how these resident strains survive in comparison to clinical and veterinary isolates.

The aim of the work in this chapter therefore was to identify parameters known to influence bacterial survival in the food factory environment; and to define environmental factors such as temperature and humidity. Stainless steel coupons were inoculated and stored at 10°C, 25°C and 37°C with varying Relative humidity (RH) levels. Once the cells could no longer be cultivated on nutrient agar, the ability of the strains to enter the VBNC phase was investigated. The growth of the strains was also investigated in both nutritious and minimal media, at 10°C, 25°C and 37°C.

4.2 Methods and materials

4.2.1 Microorganisms

Salmonella Typhimurium, *Salmonella* Senftenberg, *Salmonella* Livingstone, *Salmonella* Kedougou, *Salmonella* Montevideo, *Salmonella* Schwarzengrund and were stored on Microbank beads (Fisher Scientific, UK) and maintained at -80°C until required. The surface of a nutrient agar plate was inoculated with a single bead and incubated for 18 hours at 37°C in aerobic conditions. A single colony from the Nutrient agar plate was selected using a sterile inoculating loop and 10mls of Nutrient broth (Oxoid, UK) was inoculated and incubated at 37°C in a shaking incubator for 18 hours.

4.2.2 Microbiological Media

Nutrient agar and Nutrient broth was supplied by Oxoid Ltd (UK) and prepared by following manufacturer's instructions and autoclaved at 121°C for 15 minutes. The agar was allowed to cool down to 5°C and poured into sterile Petri dishes (Sarstedt Ltd, Leicester,UK). Sodium chloride was purchased from Fisher Scientific (UK), saline was prepared by adding 8.5g of sodium chloride to 1L water to make a 0.85% solution and autoclaved. Minimal media (M9 salts) was purchased from Sigma Life science (UK) and prepared by adding 56.4g of powder to 1L water and autoclaved. A 5x concentrated stock solution was prepared by suspending 56.4g powder in 1L water, this was autoclaved for 15 minutes at 121°C to sterilize. the 5x M9 stock solution was diluted to a 1x working solution by adding 100mL of 5x M9 stock to 400mL sterile water and allowed to cool before the addition of 0.3g of glucose powder to gain a 0.6% glucose enriched medium (Sigma Aldrich,UK). Luria broth was also purchased from Sigma Life science and prepared by suspending 15.5g powder in 1L water, this was then autoclaved for 15 minutes at 121°C to sterilize.

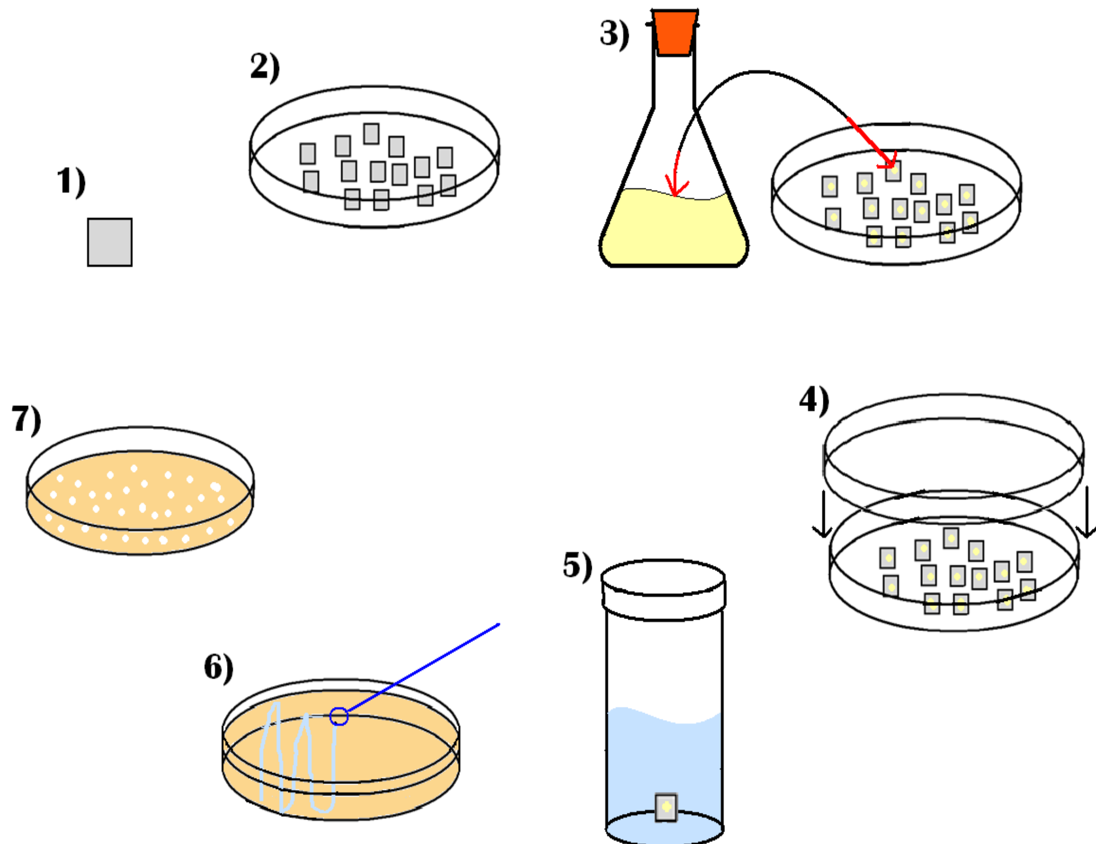
4.2.3 Steel sources

All steel used in the bacterial survival studies was purchased online from the company Metals4U (West Yorkshire, UK). The stainless steel sheet contained approximately 18% chromium which gave it better resistance against heat and corrosion. The stainless steel sheet was 0.9mm thick. A stock of $10mm^2$ coupons were cut from the master sheet using a guillotine, placed in a 100ml glass Duran bottle and sterilised by autoclaving at 121°C for 15 minutes. For the bacterial survival studies on steel the method described in figure 35 was used and repeated for all ten isolates of *Salmonella* at the three temperatures.

4.2.4 Temperature selection

Temperature and RH data for a period of 11 days was provided by a Mars factory branch (Peterborough, UK). As part of internal quality control within the factory a temperature and RH data logger was used to record environmental data for the sample preparation and packaging zone over a period of 11 days. The data revealed the lowest temperatures recorded were between 11°C to 15°C, during operation the maximum temperature reached in these zones was 25°C. The relative humidity levels fluctuated from 37% to 75%. In light of this information three temperature/humidity combinations were selected which consisted of 37°C with 20% RH, 25°C with 15% RH and 10°C with 70% RH. To mimic human body temperature, 37°C was selected.

Figure 35 Schematic of survival on steel experiment



1) Steel coupons were cut to size ($10mm^2$) using a hydraulic guillotine (School of Engineering, Aston University) placed in a glass universal tube and sterilised by autoclaving 121°C for 15 minutes. (2) Following sterilization coupons were then placed in a sterile Petri dish using sterilised forceps. (3) The coupons were then inoculated with 10µl of approximately 10^8 cfu/ml of a nutrient culture previously incubated at 37°C for 18 hours. (4) The inoculated coupons were placed in an incubator to dry for 60 minutes at 37°C. (5) Following drying the coupons were stored in the sterile Petri dish at the designated temperatures, at each sample time point a single disc was removed and cultured to determine surviving bacteria. This was achieved by adding each disc to 10ml of 0.085% saline solution and mixed by vortexing for 2 minutes. This was then designated the neat solution. Ten- fold dilutions were performed as required from the neat solution by taking 1ml of the solution and adding to 9ml saline until a 10^{-4} dilution was achieved. A 100µl volume of this diluted solution was then inoculated onto nutrient agar plates (6). Following 18-24 hours incubation at 37°C viable counts were recorded (7). This procedure was repeated weekly for a period of 7 weeks.

4.2.5 *Quality Control*

At each time point the steel experiment was repeated with a blank disc as a negative control. All test plates were incubated at 37ºC for 18 hours, the colony forming (CFU) units were counted and the results were recorded in tables. Furthermore all experiments were conducted in triplicate.

4.2.6 *Determination of Viable Non Culturable Cells by BacLight staining*

Fluorescence microscopy (Zeiss Axioskop) of the test strains previously inoculated onto stainless steel coupons was achieved by using the LIVE/DEAD® BacLight™ Bacterial Viability Kit purchased from Life Technologies (UK). The stain was stored in the – 20°C freezer and before use the stain was allowed to stand at room temperature for 30 minutes.
Equal volumes (2µl) of SYTO® 9 and propidium iodide were combined in a microfuge tube and mixed thoroughly by using a pipettor. A 200µl volume of sterile water was adding to the microfuge tube and mixed by vortexing for 2 minutes. The preparation was left to stand for 20 minutes at room temperature. Steel coupons were fixed onto glass microscope slides and placed in sterile Petri dishes, 20µl of the solution was used to inoculate each steel disc and incubated for 30 minutes at 37°C. Following incubation any unbound dye was removed using a sterile pipette, a glass coverslip was mounted onto the steel disc using the mounting oil provided and the cells were observed under the x100 oil emulsion objective of the Zeiss light microscope. All images were saved as JPEG files.

4.2.7 *Growth curves-using the Biotek Microplate Reader*

Growth curves for *Salmonella* were produced using the Biotek's ELx808 Absorbance Microplate reader, running Gen5™ software to automatically log and save absorbance data. A 96 well, flat bottom plate was used, as the reader is at the bottom of the microplate it is not able to read through round wells. The multi-well plate was pre-labelled and 198µl of the broth being tested was dispensed into each well. Next, 2µl of the overnight cultures was added into each well and mixed by inverting up and down 5 times. The last 2 wells in each row were selected as controls containing only nutrient broth. Parafilm (Appleton Woods, UK) was used to seal the top of the plate to prevent spillage in the reader. The plate was then placed in the absorbance reader and the programme was set; the plate was run for 72 hours for nutrient

broth (30 hours for Minimal media and Luria broth). The temperature was selected and the absorbance reader was set to take a reading every hour at 570nm. As the plate reader does not have a cooling function, for the 10°C growth curves in nutrient media, the absorbance reader was placed in an environmental chamber in the engineering department of Aston university. The chamber artificially replicates conditions and allows the temperature and RH to be controlled, the temperature in the chamber was programmed to 8°C, creating a lower ambient temperature, this allowed the temperature of the Microplate to reach 10°C. Following completion the data from the Gen5™ software were exported into a Microsoft excel file and converted into graphs.

Figure 36 A 96 well micro titre plate placed in the ELx808 absorbance reader, which runs on the Gen5 programme on the PC

4.2.8 Data preparation and analysis

Survival data were converted into log CFU/ml and used to produce line graphs, a line of best fit was added to each curve. From this data, the Decimal reduction Time (DRT) was calculated, in microbiology this is defined as the time taken for a population of organisms to reduce by one log order (90%) at a fixed temperature. The DRT values can be used to predict survival/death of an organism even after sampling has stopped. The absorbance data for the *Salmonella* growth curves in both nutritious and minimal media was exported into the statistical software Statistica (version 10, USA) and a Repeated Measures Anova (RMANOVA), was selected to highlight any potential differences across strains and to ensure the technical repeats were in line.

Figure 37 Survival of *Salmonella* at 10°C on stainless steel

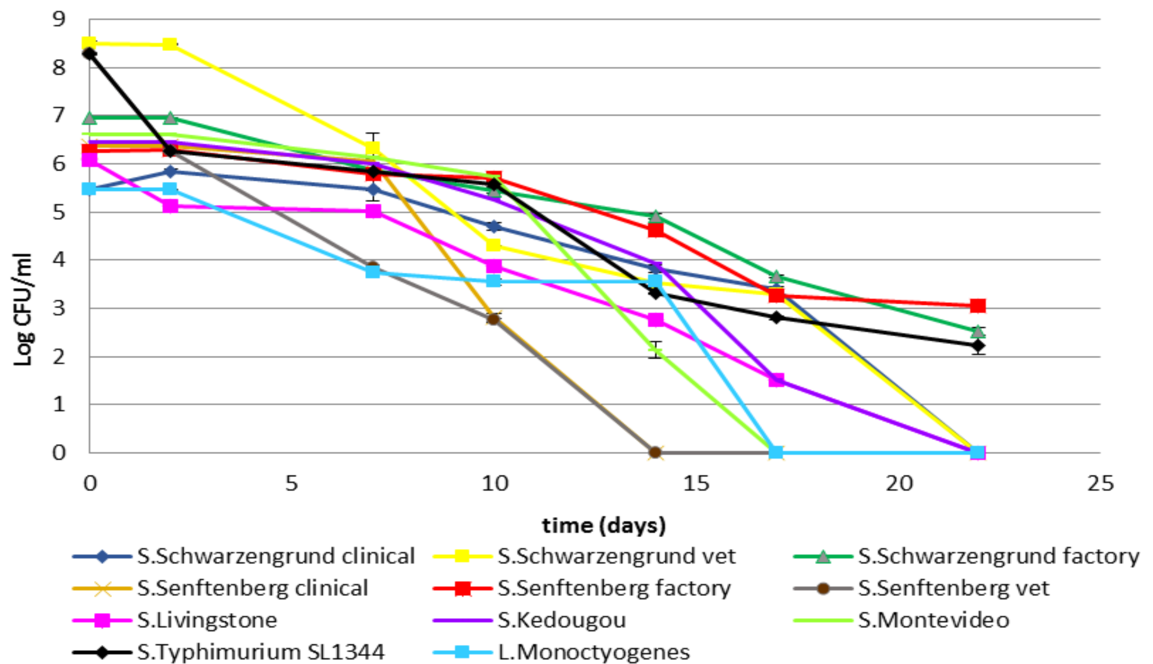


Figure 37: shows survival of a panel of factory, clinical and veterinary strains of *Salmonella* and *L.monocytogenes* at 10°C on stainless steel. D values were calculated by subtracting time at log 6 from time at log 5. From a starting inoculum of between 10^5 and 10^8 at time 0, the clinical isolate of *S*.Schwarzengrund and the factory isolate of *S*.Senftenberg, only demonstrated a 1.5 to 1.7 log reduction in population number over 14 days. The Factory isolates of *S*.Kedougou, *S*.Livingstone and *S*.Schwarzengrund demonstrated a 2- 3.3 log reduction in population number. Whereas the factory isolate of *S*.Montevideo, the veterinary isolate of *S*.Schwarzengrund, the clinical isolate of *S*.Senftenberg, and *S*.Typhimurium SL1344 demonstrated a 4.5 to 6.4 log reduction in population number. The veterinary isolate of *S*.Senftenberg revealed an 8 log reduction over 14 days. A 2-3 log survival was observed for factory isolates of *S*.Senftenberg, *S*.Schwarzengrund and *S*.Typhimurium after 22 days, whereas the remainder of isolates showed no survival. An independent reading was taken at 72 days which showed <10 cfu ml^{-1} survival across all strains. Error bars on the graph represent standard deviation, all error bars were small indicating that values obtained were close to the mean and the data points were not spread out, with the exception of *L.monocytogenes* for which large error bars were obtained at 7 days. Following the 72 day read all coupons were tested for cells that may possibly be entering the VBNC state using BacLight staining kit.

Figure 38 Images taken under fluorescence microscopy using BacLight stained samples following 72 days incubation at 10°C

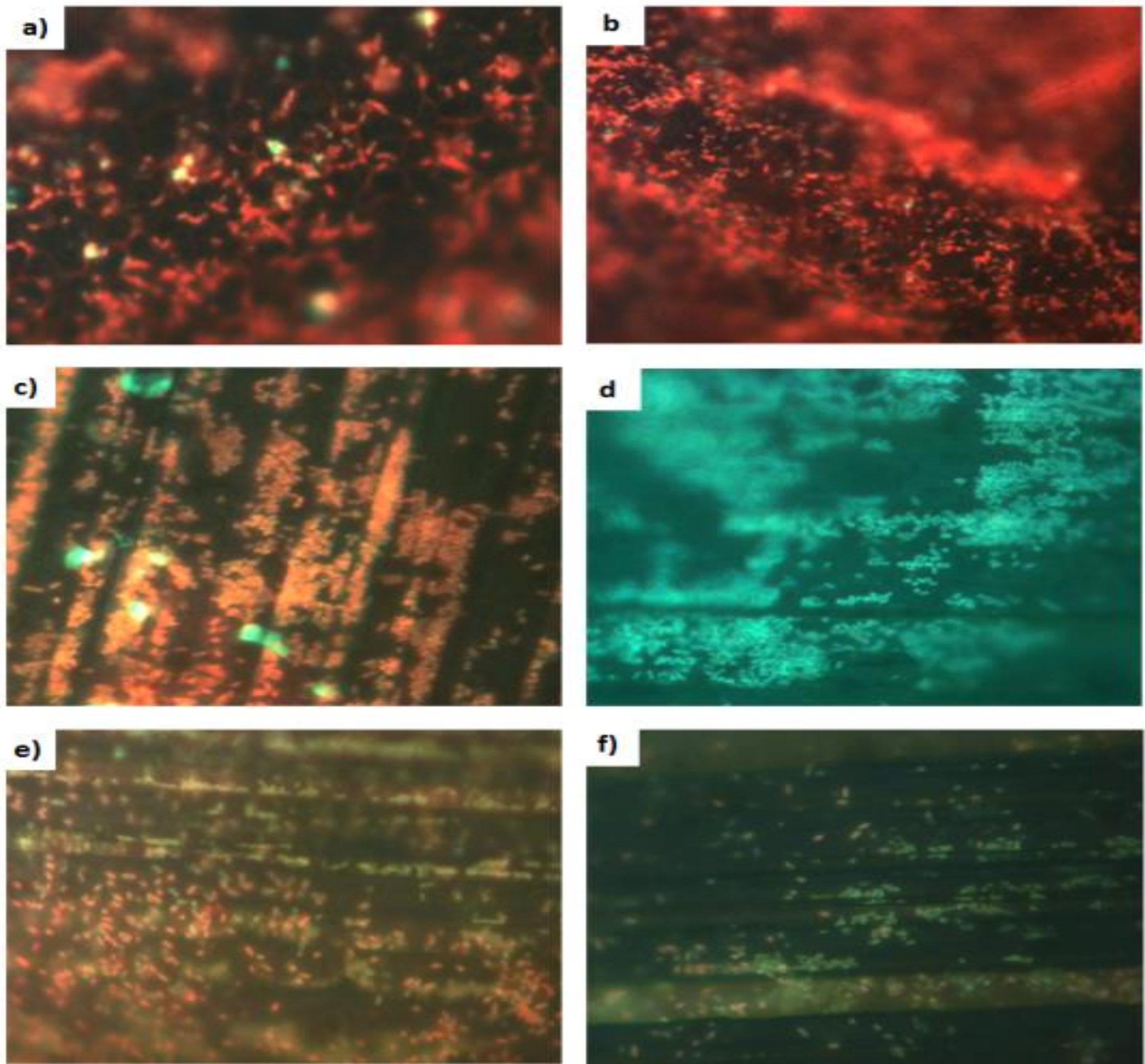


Figure 38 Images taken under fluorescence microscopy using Baclight stained samples Following 72 days incubation at 10°C when cfu data was <10 cfu ml^{-1}. a) *S.*Senftenberg factory b) *S.*Senftenberg clinical c) *S.*Senftenberg vet d) *S.*Schwarzengrund factory e) *S.*Schwarzengrund clinical f) *S.*Schwarzengrund vet. The vivid green cells in d) suggest that the factory strain of *S.*Schwarzengrund is completely excluding the propidium iodide stain and only taking up the STYTO 9 meaning these cells are entering the VBNC state. Images e&f) show a proportion of green cells and a proportion of red cell, indicating that the clinical and veterinary isolates of *S.*Schwarzengrund may also be entering this state.

Figure 39 Images taken under fluorescence microscopy using BacLight stained samples following 72 days incubation at 10°C

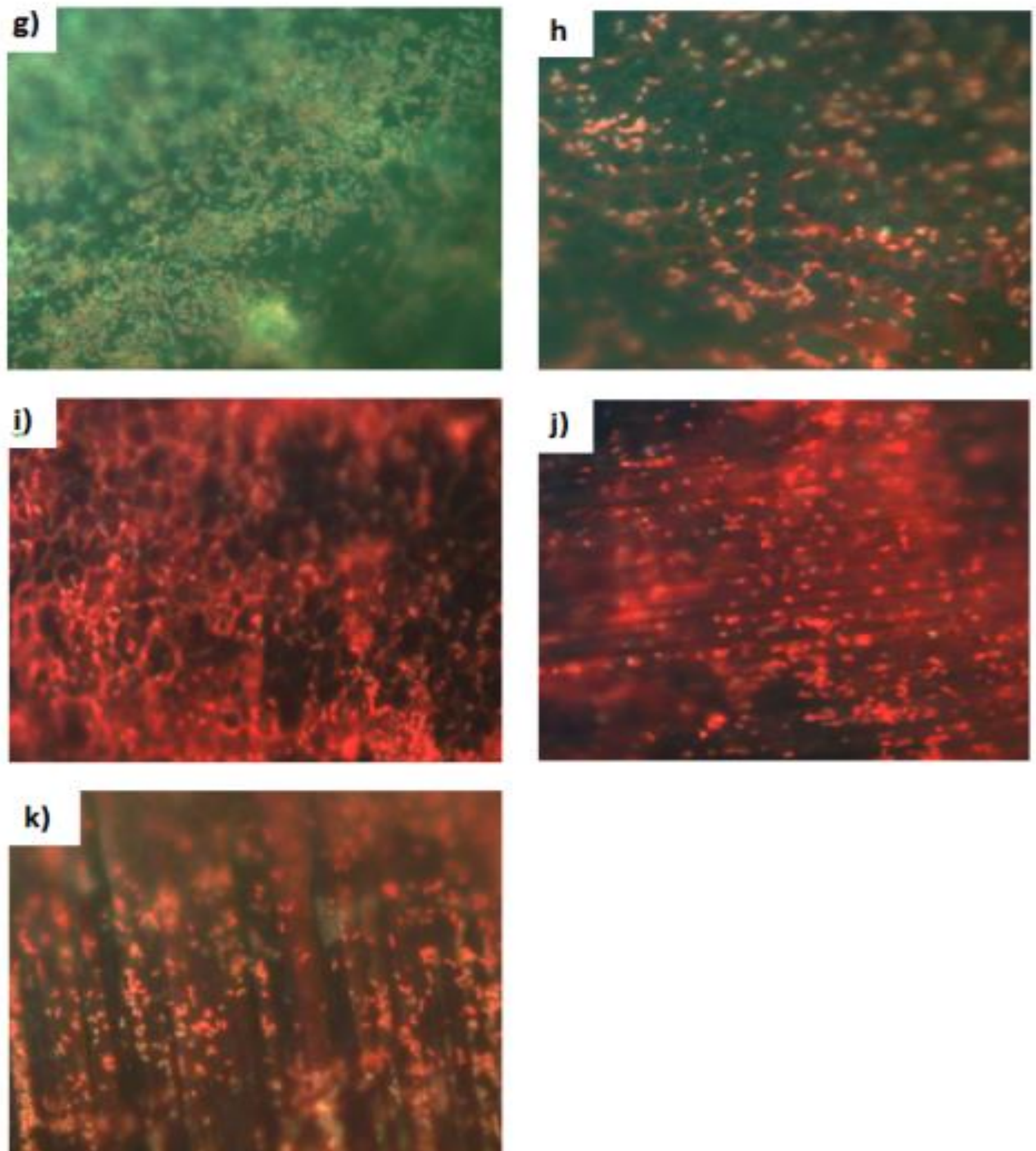


Figure 39: images taken under fluorescence microscopy using BacLight stained samples following 72 days incubation at 10°C when cfu was <10 cfu ml^{-1}. g) *S.*Livingstone factory h) *S.*Montevideo factory, i) *S.*Kedougou factory j) *S.*Typhimurium SL1344, k) *L.monocytogenes.* All strains appear to be taking up the propidium iodide stain and reducing SYTO®, with the exception of *S.*Livingstone, which appears to have some green cells.

Figure 40 Survival of *Salmonella* on stainless steel at 25°C

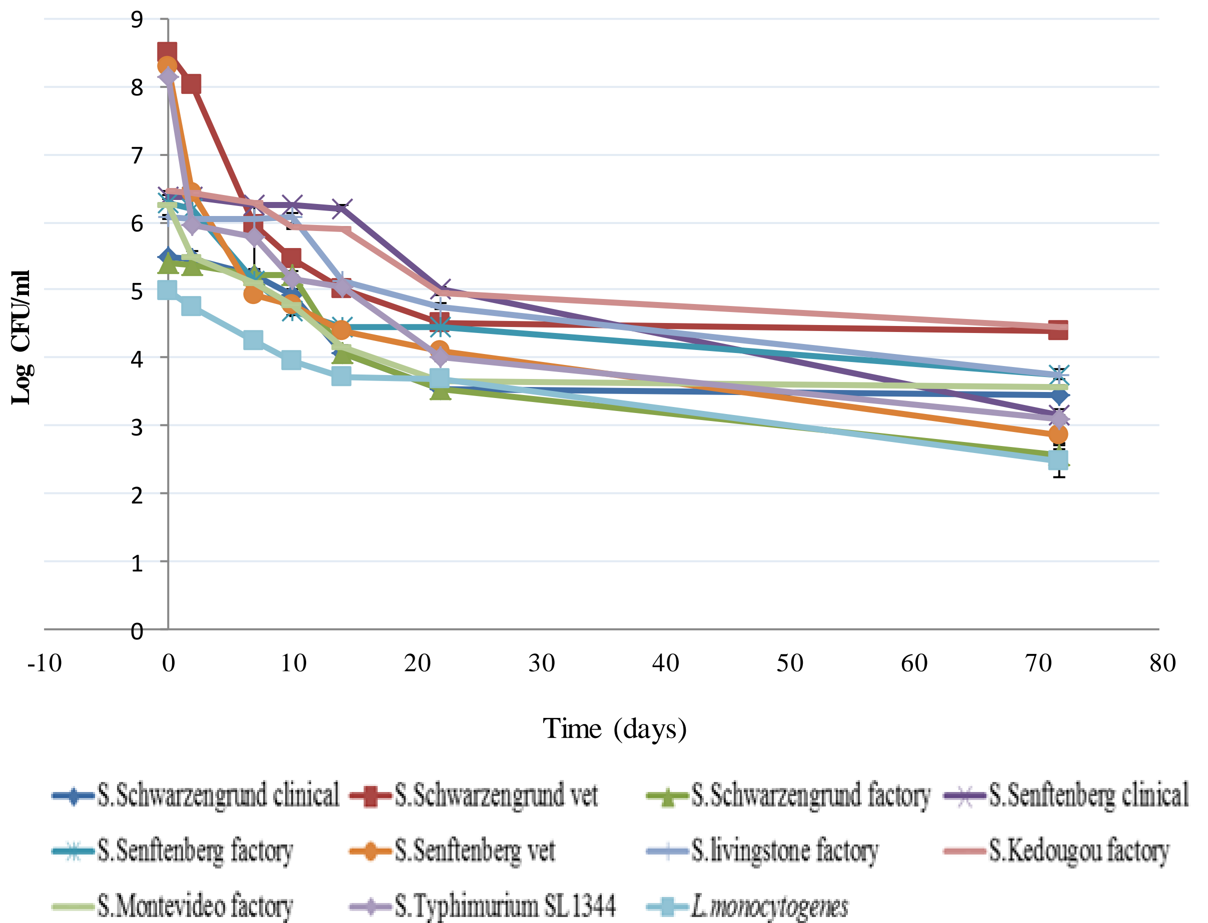


Figure 40: shows survival of a panel of factory, clinical and veterinary strains of *Salmonella* isolates and *L.monocytogenes* at 25°C on stainless steel. Samples were taken twice a week for 22 days and an independent sample was taken after 72 days. From a starting inoculum of between 10^5 and 10^8 at day 0 a majority of the strains regardless of serotype and origin demonstrated a 1 to 2 log reduction in population number over the first twenty two days, with the exception of both the veterinary isolate of *S.*Schwarzengrund and *S.*Senftenberg, as well as *S.*Typhimurium SL1344 which revealed a 4 log reduction in population number over the first twenty two days. All of the isolates settled around a log 3 to 5 surviving number after 72 days of incubation. All error bars were small indicating that values obtained were close to the mean and the data points were not spread out. At this point all coupons were stained using the BacLight stain to firstly visualise if cells were viable and secondly to verify the CFU data.

Figure 41 images taken under fluorescence microscopy using BacLight stained samples following 72 days incubation at 25°C

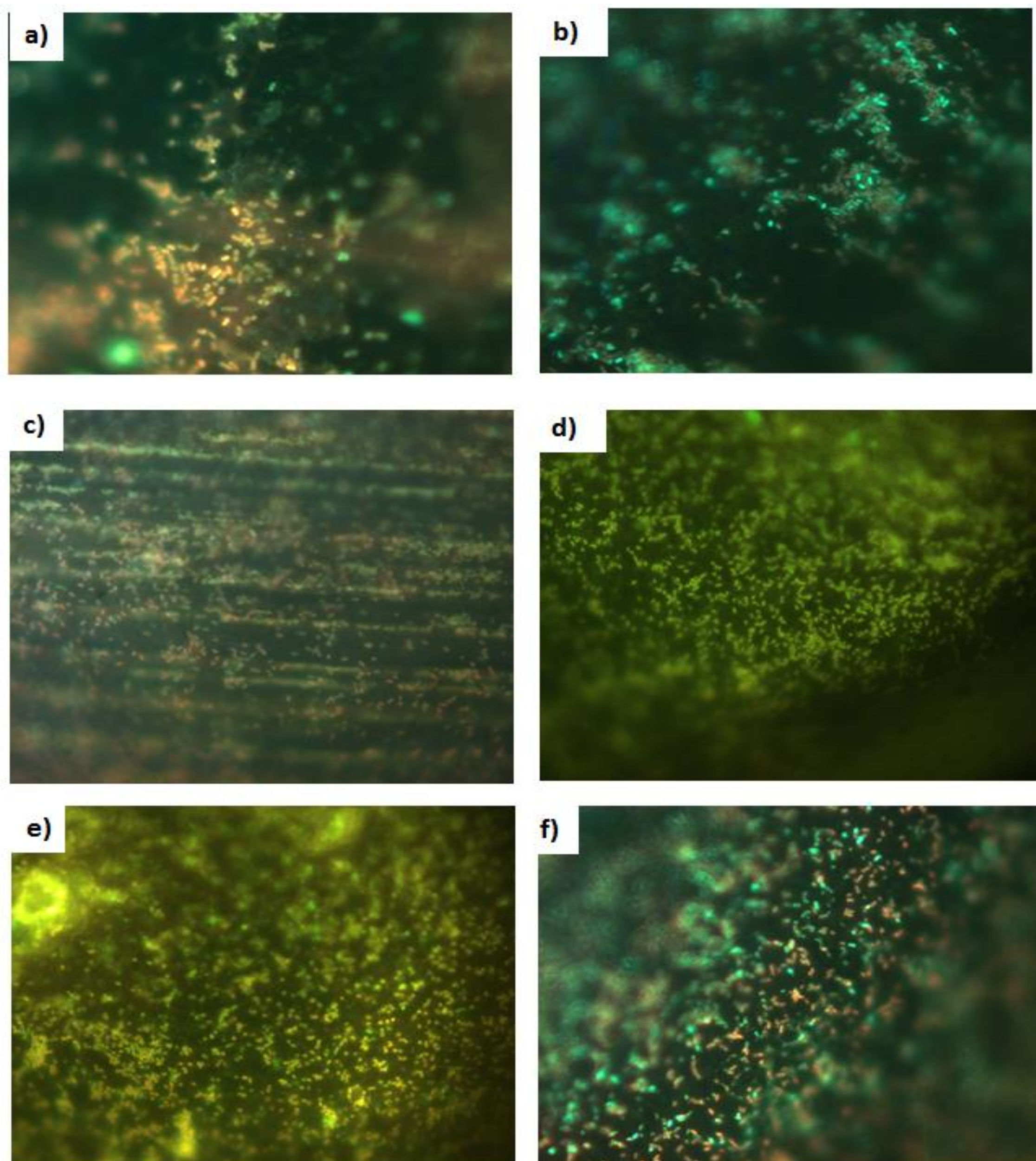


Figure 41: images taken under fluorescence microscopy using BacLight stained samples following 72 days incubation at 25°C a) *S.*Schwarzengrund factory b) *S.*Schwarzengrund clinical c) *S.*Livingstone factory d) *S.*Senftenberg factory e) *S.*Senftenberg clinical f) *S.*Senftenberg vet. The images correspond to cfu data, which showed 3-5 log survival, there is a combination viable and dying cells; a majority of the cells appear green, meaning they are excluding the propidium iodide stain whereas some cells are an orange-red in colour and are therefore reducing SYTO® 9.

Figure 42 Survival of *Salmonella* on stainless steel at 37°C

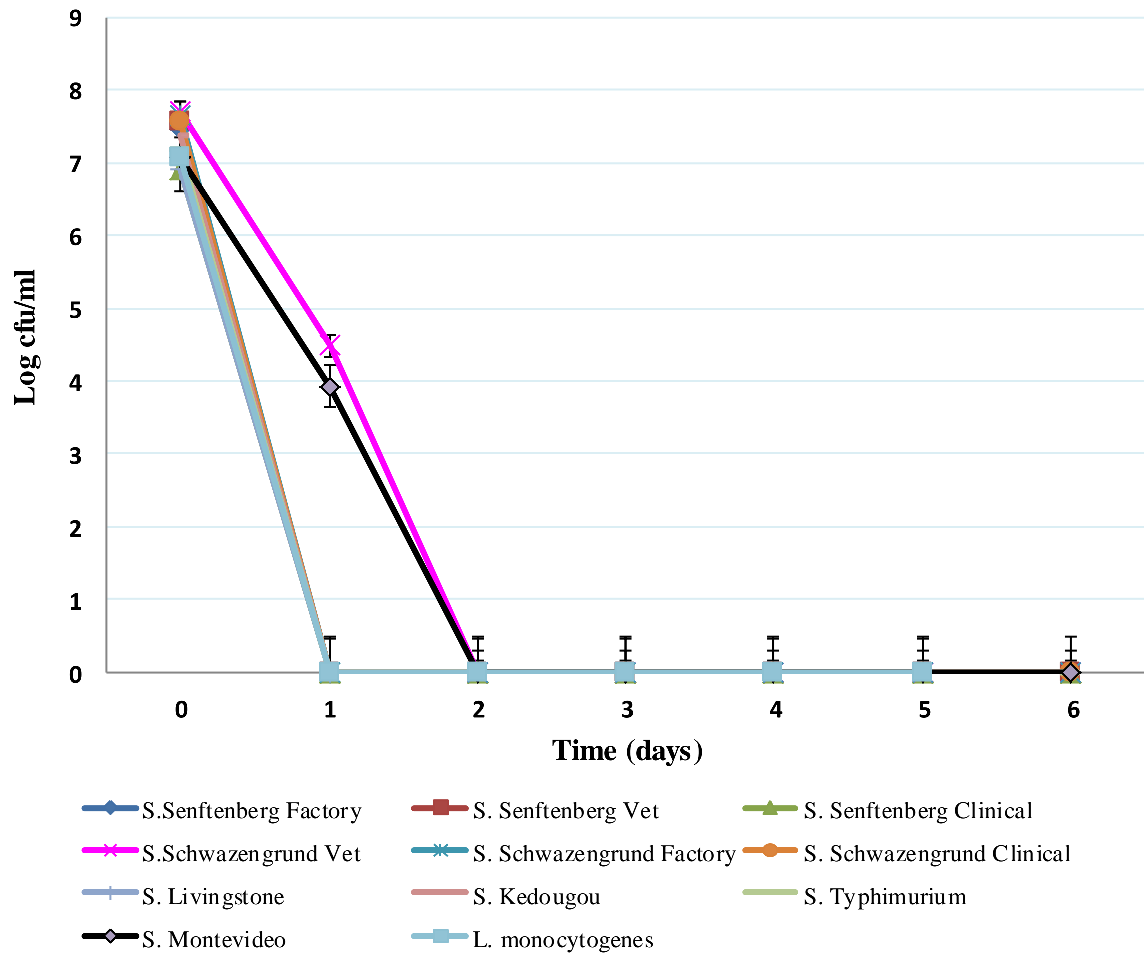


Figure 42: For inoculated coupons stored at 37°C, samples were taken at time 0, 1 day. 2 days, 3 days, 4 days, 5 days and 6 days. However, after 24 hours a majority of the agar plates showed <10 cfu ml^{-1} with the exception of the factory isolate of *S*.Montevideo and the veterinary isolate of *S*.Schwarzengrund which demonstrated <0 cfu/ml after 48 hours. Due to time constraint it was not possible to take multiple readings in between 0 and 24 hours. The error bars represent standard deviation, all error bars were small indicating that values obtained were close to the mean and the data points were not spread out. At both time 0 and 48 hours all coupons were stained using the backlight to verify the CFU data.

Figure 43 Images taken under fluorescence microscopy using BacLight stained samples at 37°C:

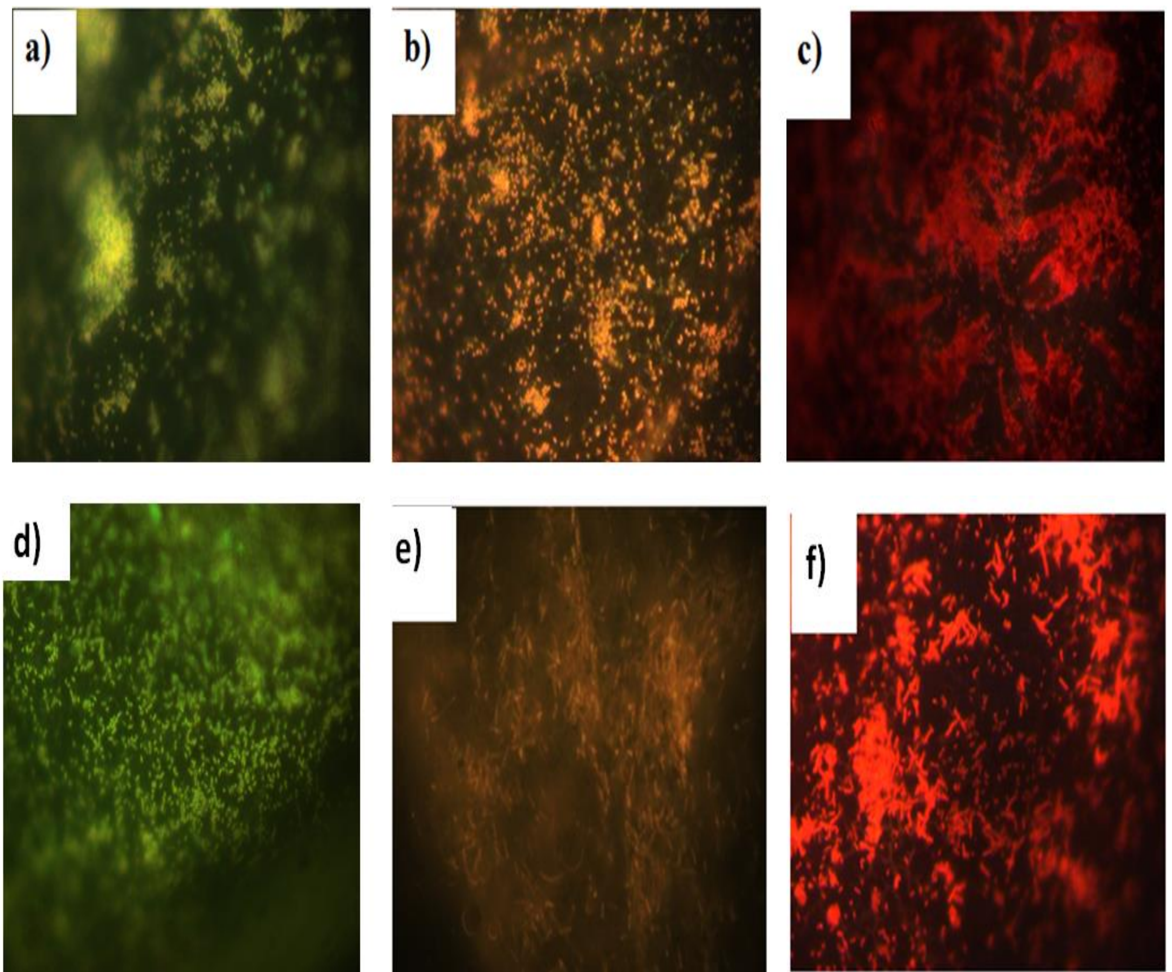


Figure 43: Images taken under fluorescence microscopy using BacLight stained samples at 37°C; after 24hours no colony forming units were visualised on the agar plates, to observe if isolates were entering the VBNC state images were acquired at time 0 following drying and 24 hours when cfu data was <10 cfu ml-1. a) *S.*Livingstone time 0 b) *S.*Livingstone time 24hours c) *S.*Livingstone time 48 hours d) *S.*Kedougou time e) *S.*Kedougou 24hours f) *S.*Kedougou at 48 hours At time 0, green cells can be observed this reflects the microbiology data which show the cells were viable and culturable. The distinctive red after 48 hours indicates that cells are taking up the propidium iodide stain and reducing SYTO® 9 indicating at 37°C they are dead/dying and are not entering the VBNC phase.

Figure 44 Images taken under fluorescence microscopy using BacLight stained samples at 37°C

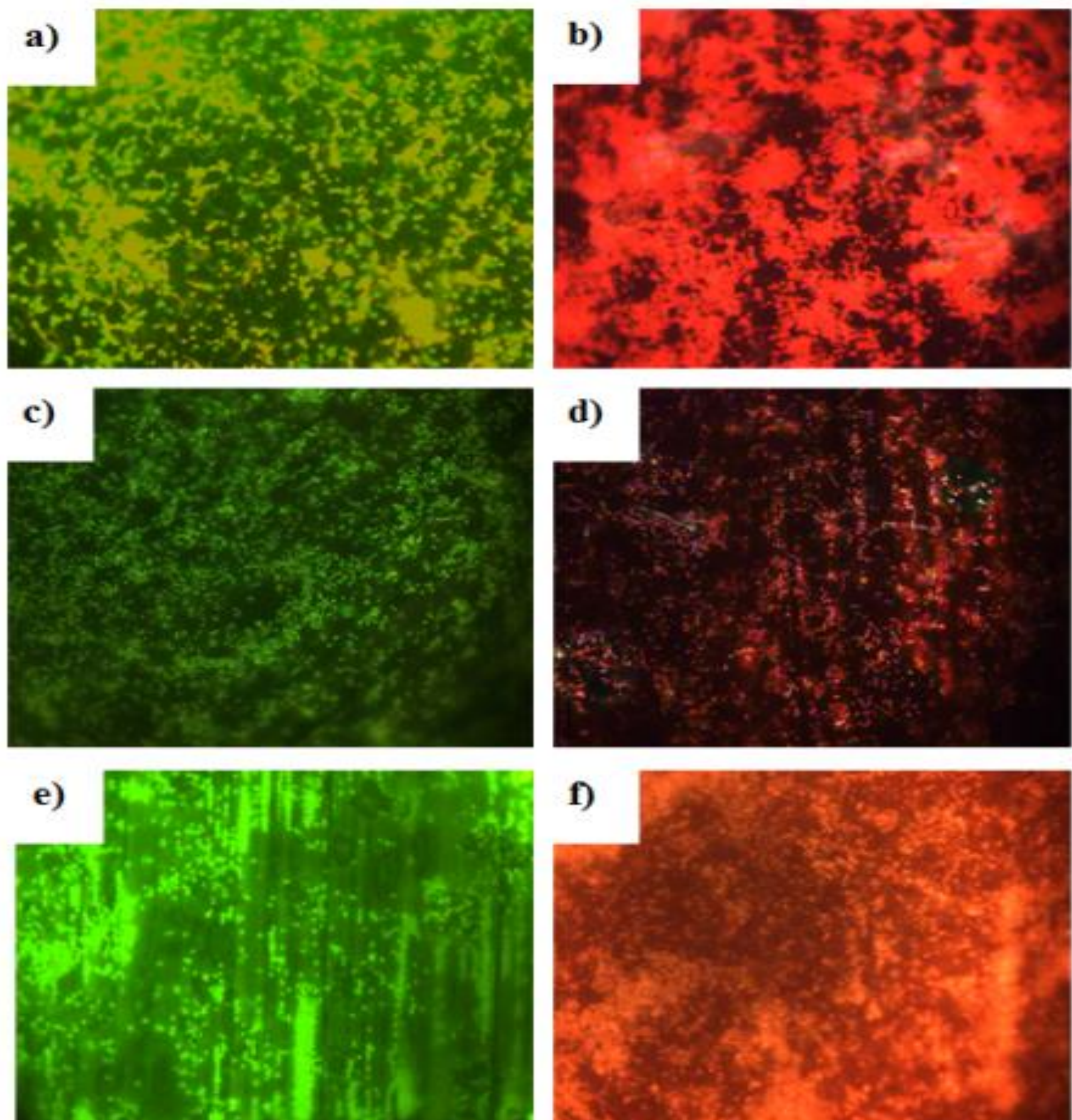


Figure 44: Images taken under fluorescence microscopy using BacLight stained samples at 37°C; after 24hours no colony forming units were visualised on the agar plates, to observe if isolates were entering the VBNC state images were acquired at time 0 following drying and 48 hours when cfu data was <10 cfu ml^{-1} a) *S.*Schwarzengrund factory time 0 b) *S.*Schwarzengrund factory time 48hours c) *S.*Schwarzengrund clinical time 0 d) *S.*Schwarzengrund clinical time 48hours e) *S.*Schwarzengrund vet at time 0 f) *S.*Schwarzengrund vet at time 48hours. The distinctive red colour indicates that the cells were taking up the propidium iodide stain and reducing SYTO® 9 this reflects the microbiology data which show the cells were not culturable after 48 hours

Figure 45 Images taken under fluorescence microscopy using BacLight stained samples at 37°C

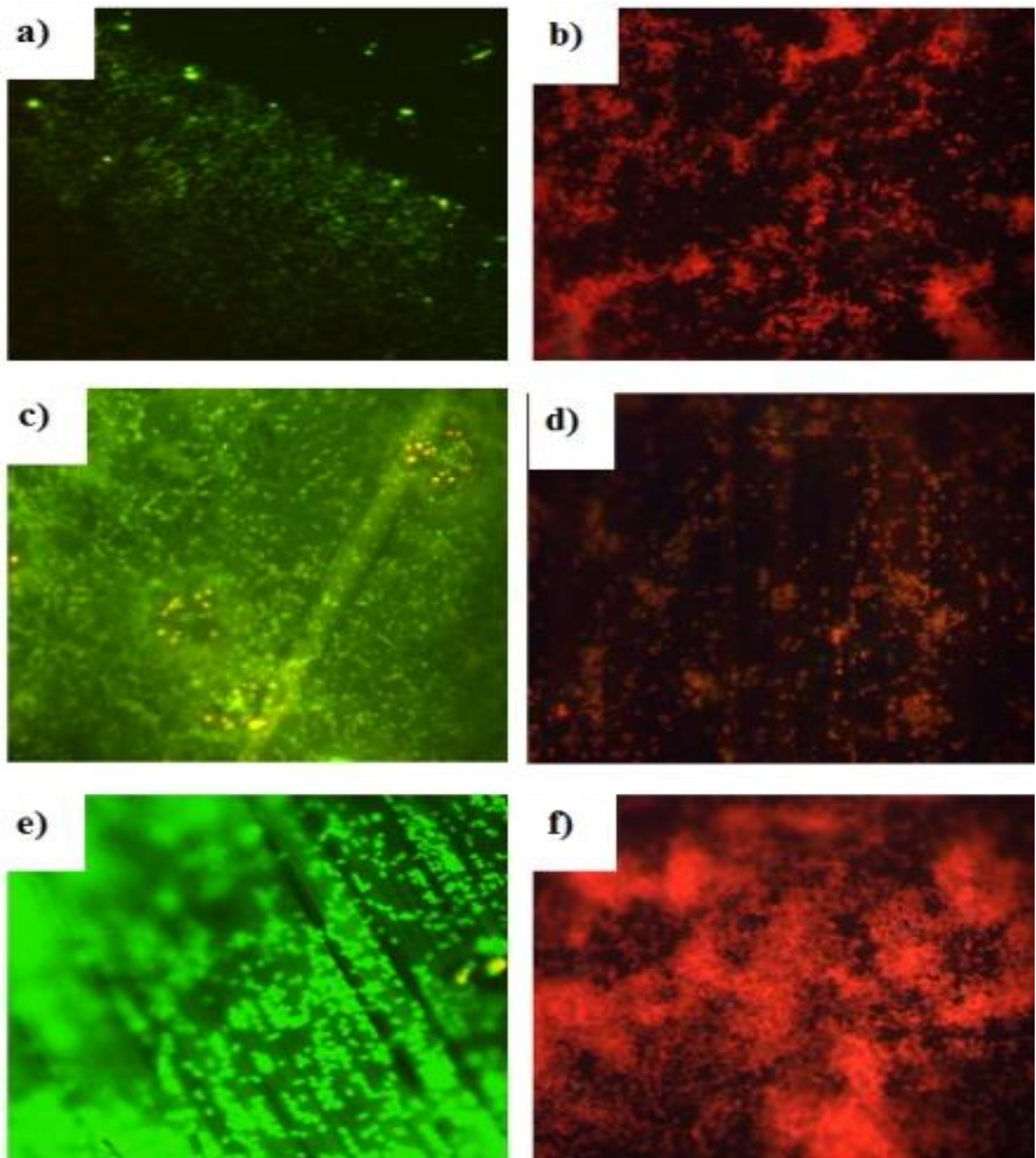


Figure 45 : Images taken under fluorescence microscopy using BacLight stained samples at 37°Ca) *S.*Senftenberg factory time 0 b) *S.*Senftenberg factory time 48hours c) *S.*Senftenberg vet time 0 d) *S.*Senftenberg vet time 48hours e) *S.*Senftenberg clinical time 0 f) *S.*Senftenberg clinical time 48hours The distinctive red after 24hours indicates that cells are taking up the propidium iodide stain and reducing SYTO® 9. This reflects the microbiology data which show the cells were not culturable after 48 hours

Figure 46 Images taken under fluorescence microscopy using BacLight stained samples at 37°C

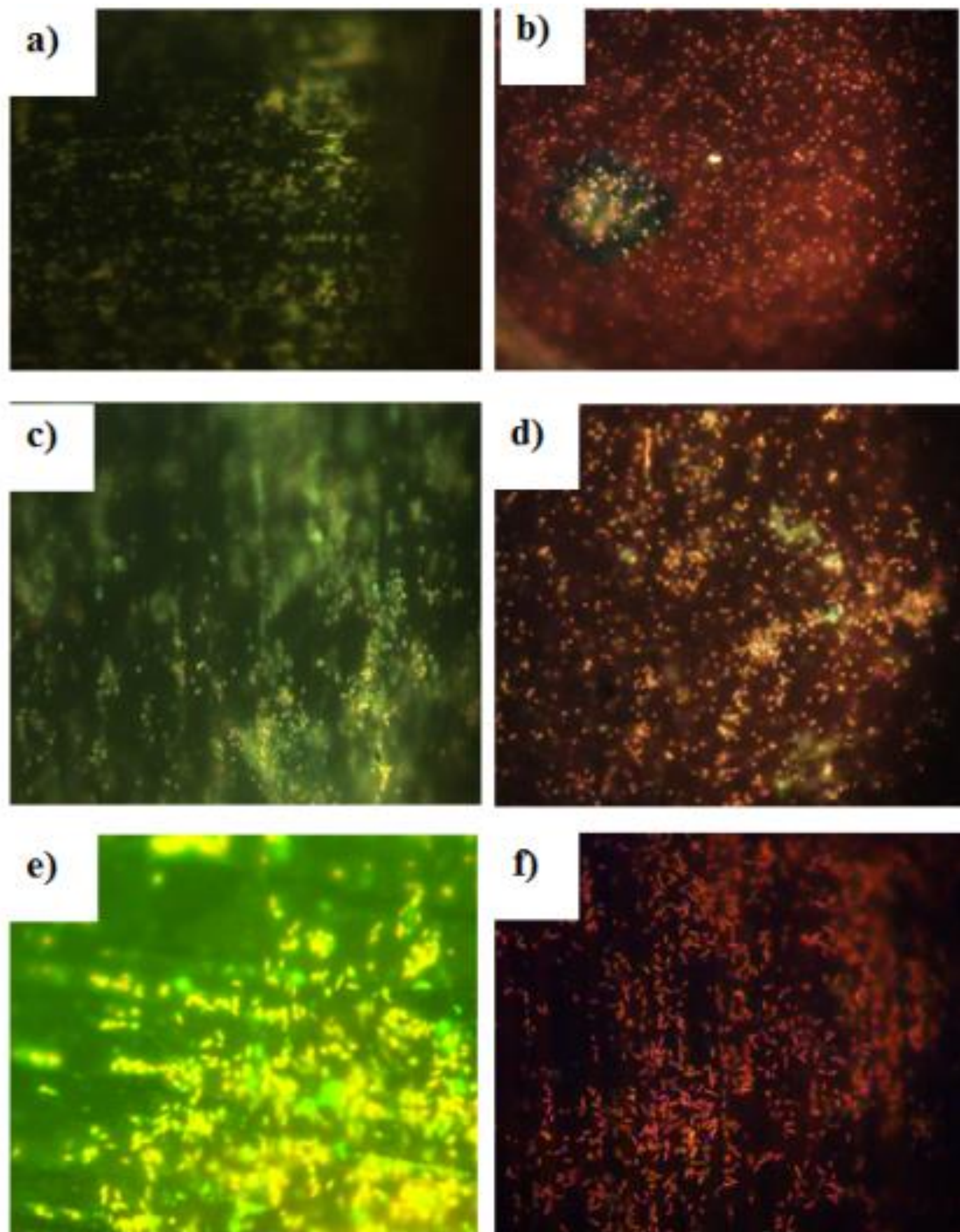


Figure 46 : images produced using BacLight stain at 37°C a) *S.*Typhimurium time 0 b) *S.*Typhimurium time 48hours c) *S.*Montevideo 0 d) *S.* Montevideo time 48 hours e) *L.monocytogenes* at time 0 f) *L.Monocytogenes* at time 48hours. Although there is a lot of background stain there in the mages, the colour of the cells can clearly be visualised. The distinctive red after 24hours indicates that cells are taking up the propidium iodide stain and reducing SYTO®.

Using the survival data, individual graphs were produced, a linear line was inserted through the points through the points, which allowed the Decimal Reduction times (D values) to be calculated for each strain. Further on, D values are expressed with the temperature as subscript (e.g. D_{10}=4.382).

Table 7 Determination of decimal reduction times (D values)

Strains	10°C/ 70% RH	25°C/ 15% RH	37°C/ 20% RH
***S*.Schwarzengrund clinical**	4.38	21.05	0.13
***S*.Schwarzengrund vet**	2.64	22.99	0.26
***S*.Schwarzengrund factory**	4.91	29.69	0.13
***S*.Senftenberg clinical**	2.20	21.05	0.15
***S*.Senftenberg factory**	6.1	33.89	0.13
***S*.Senftenberg vet**	2.02	18.87	0.13
***S*.Livingstone factory**	1.48	29.67	0.13
***S*.Kedougou factory**	3.27	35.46	0.13
***S*.Montevideo factory**	3.78	34.25	0.28
***S*.Typhimurium SL1344**	3.74	19.65	0.14
L.monocytogenes	3.74	32.47	0.14

Table 7: Decimal reduction times (D values) in days for *Salmonella* isolates and *L.monocytogenes* at 10°C, 25°C and 37°C on stainless steel coupons. Analysis reveals that at 25°C and 15% humidity, the factory strains of both *S*.Senftenberg (D=33.89) and *S*.Schwarzengrund (D=29.69) demonstrated a longer D value compared to serotype matched clinical and veterinary strains. The average D value at 25°C was D=27.2, the veterinary isolate of *S*.Senftenberg (D=18.8) exhibited the lowest D value, followed by *S*.Typhimurium SL1344 (D=19.64). Overall of the *Salmonella* strains tested the factory strains demonstrated the longest D values. At 10°C and 70% humidity, the factory strain of *S*.Senftenberg demonstrated the highest D value (D=6.1) compared to all other strains in the panel. The lowest D value was of the factory strain of *S*.Livingstone (D=1.79). The remaining D values for *Salmonella* and *Listeria* ranged between D=2.02 and D=4.91, with the average being D=3.48. At 37°C and 20% humidity, a rapid decrease was observed with all strains dead within 48 hours, A 7 log reduction was observed , equating to an average D=3.84 hours. The results indicate that high humidity (70%) coupled with low temperature resulted in a rapid decrease in cell counts.

4.4 Profiling growth in nutrient rich media

Figure 47 Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 37°C in Nutrient media

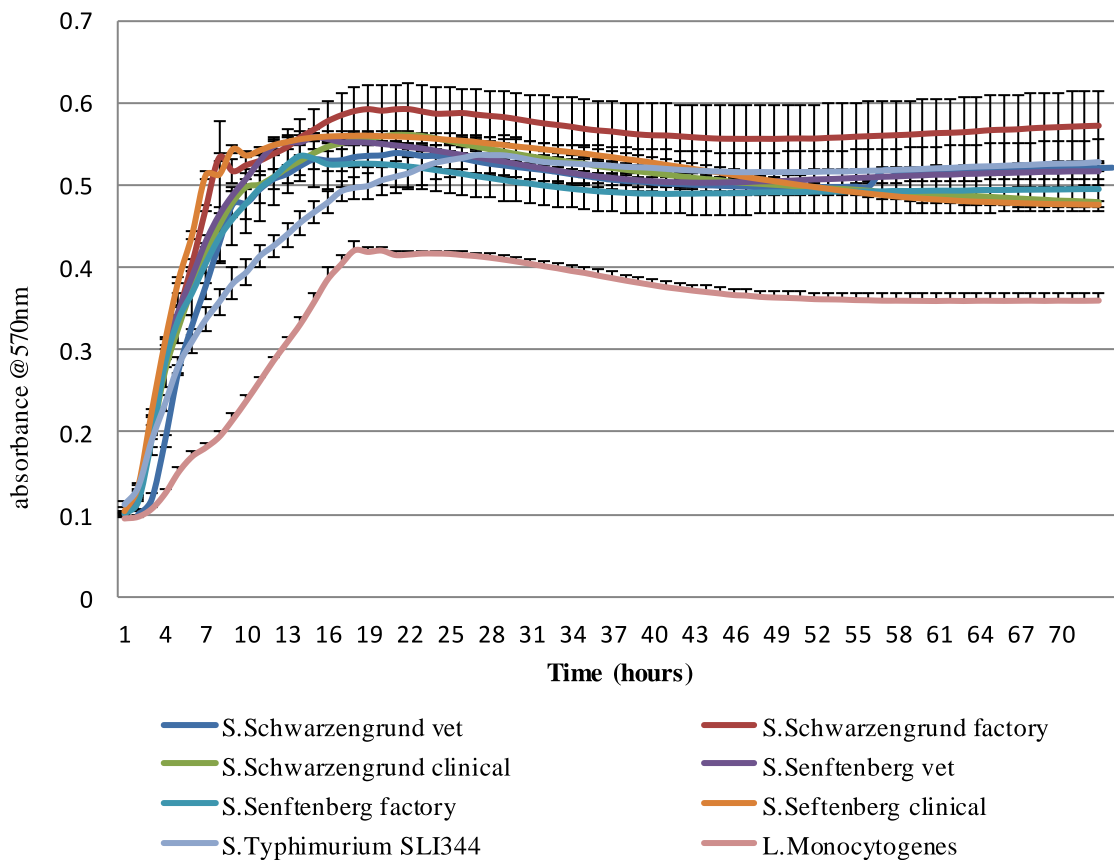


Figure 47: Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 37°C in Nutrient broth produced using the Biotek absorbance reader. This shows that all strains have the ability to grow in nutrient media with *L.monocytogenes* 11994 growing to a lower density in comparison to the *Salmonella* isolates. *Salmonella* growth ranged between 0.47 and 0.6nm, whereas *L.monocytogenes* grew to 0.42nm. The error bars represent standard deviation. This graph does not reveal any distinctive differences in the growth between the *Salmonella* isolates. Further analysis was performed using RMANOVA to reveal any significant patterns in growth between the isolates.

Figure 48 Output from the RMANOVA to reveal the overall differences in growth of isolates in nutrient media at 37°C

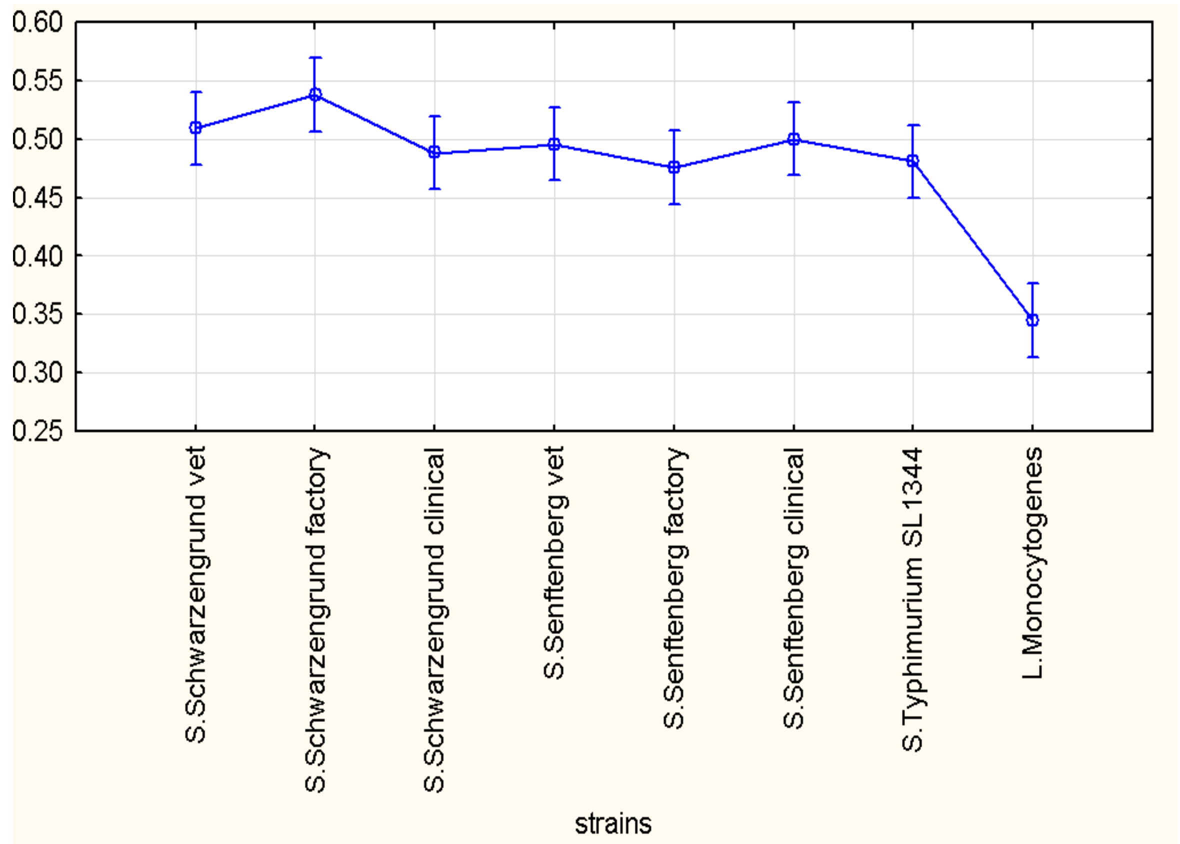


Figure 48: The Repeated measure ANOVA (RMANOVA) output shows the overall effect of growth in nutrient media on serotype at 37°C. Importantly, results indicate that there were no significant differences in the growth of factory isolates of *Salmonella* isolates however *L.monocytogenes* grew significantly slower than the *Salmonella* isolates (p=0.000). The vertical bars represent 95% confidence intervals.

Figure 49 Output from the RMANOVA to show the reproducibility of the growth curves in nutrient media at 37°C

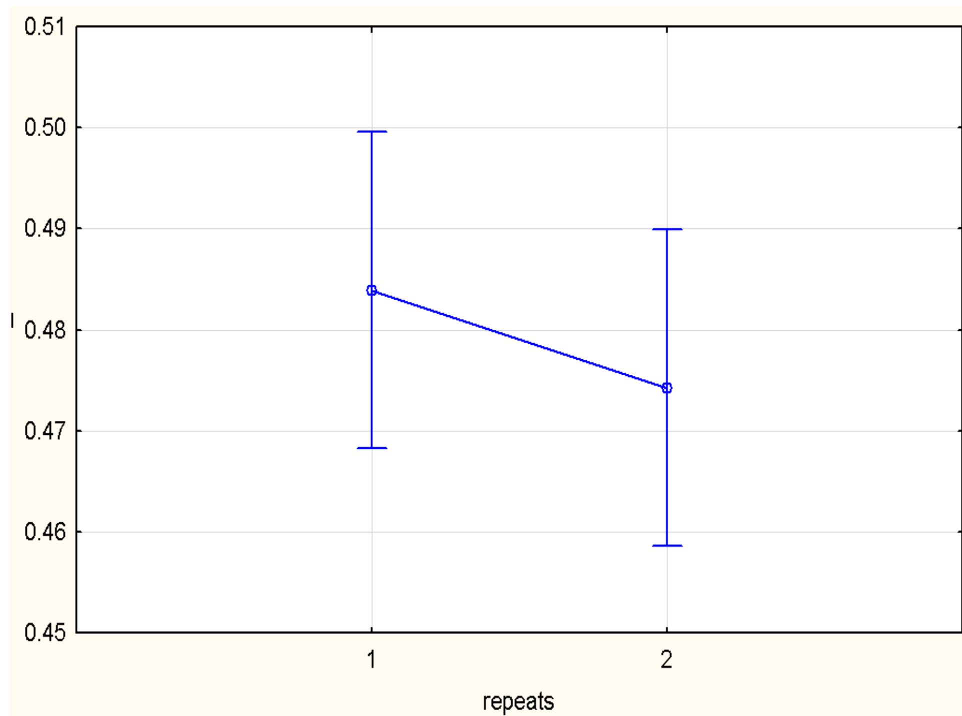


Figure 49: the output shows the two repeats were in line with each other across the strains at 37°C (p=0.3836) The vertical bars were overlapping and represent 95% confidence intervals.

Figure 50 Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 25°C in Nutrient media

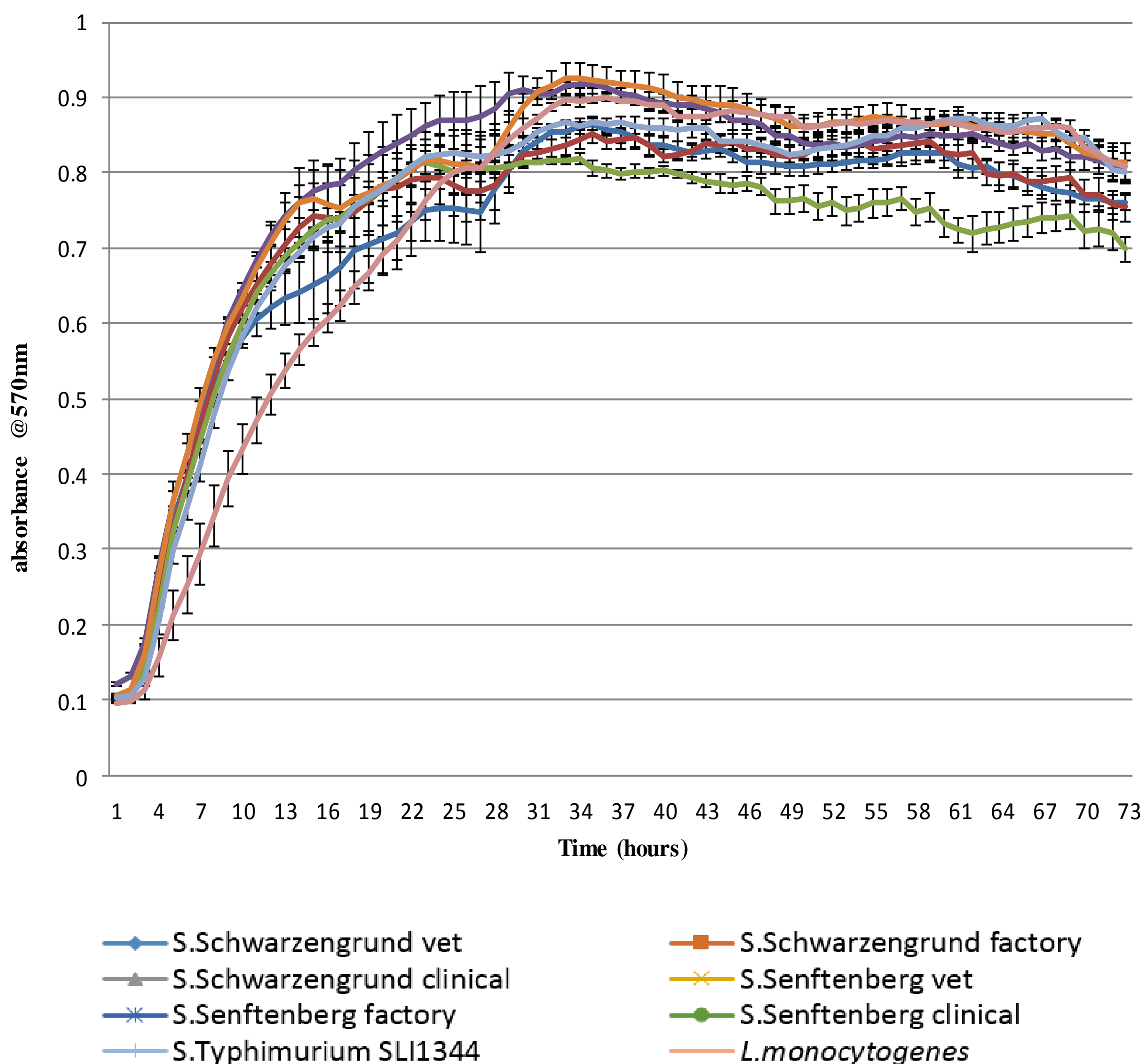


Figure 50: Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 25°C in Nutrient broth produced using the Biotek absorbance reader. This shows that all *Salmonella* strains and *L.monocytogenes* 11994 have the ability to grow in nutrient media; however the clinical isolate of *S.*Schwarzengrund seems to grow to a lower density. Average growth ranged between 0.7 and 0.9. This graph does not reveal any distinctive differences in the growth between the *Salmonella* isolates. Further analysis was performed using RMANOVA to reveal any significant patterns in growth between the isolates.

Figure 51 Output from the RMANOVA to reveal the overall differences in growth of isolates in nutrient media at 25°C

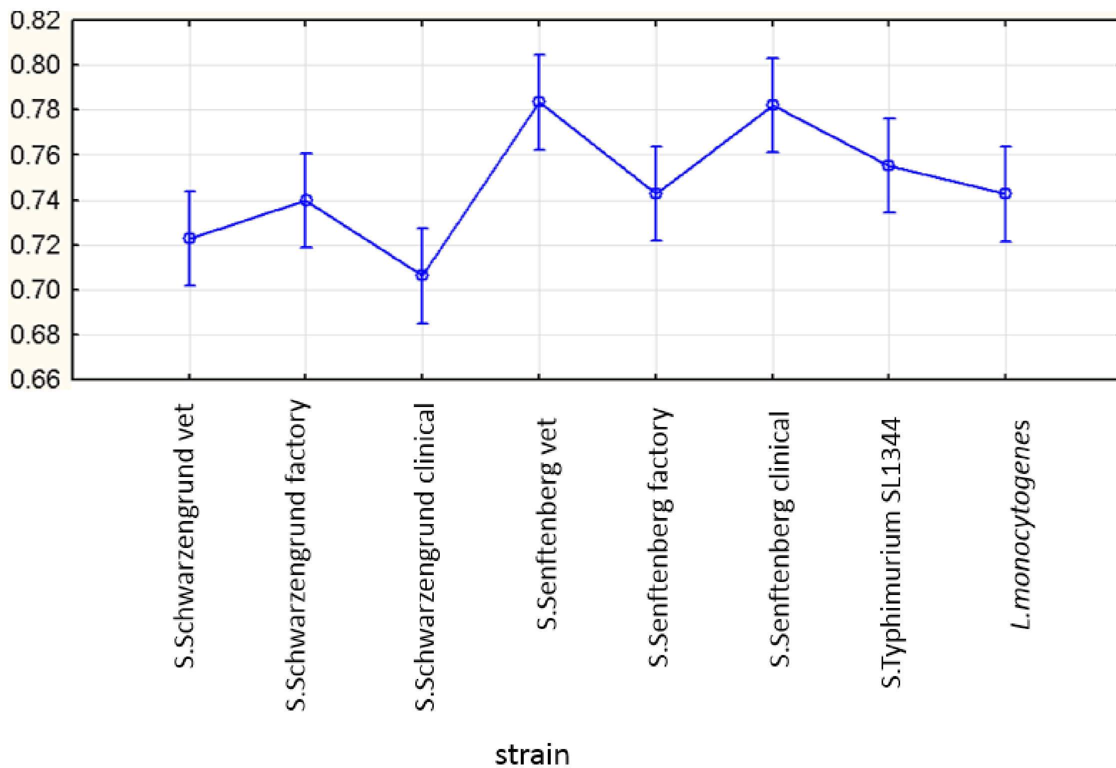


Figure 51: The Repeated measure ANOVA (RMANOVA) output shows the overall effect of growth in nutrient media on serotype at 25°C. Importantly the analysis reveals that the factory isolates do not grow significantly differently, however as a serotype the *S*.Schwarzengrund isolates seem to grow less compared to other serotypes (p=0.000). Also *L. monocytogenes* no longer out-competed at this temperature.

Figure 52 Output from the RMANOVA to show the reproducibility of the growth curves in nutrient media at 25°C

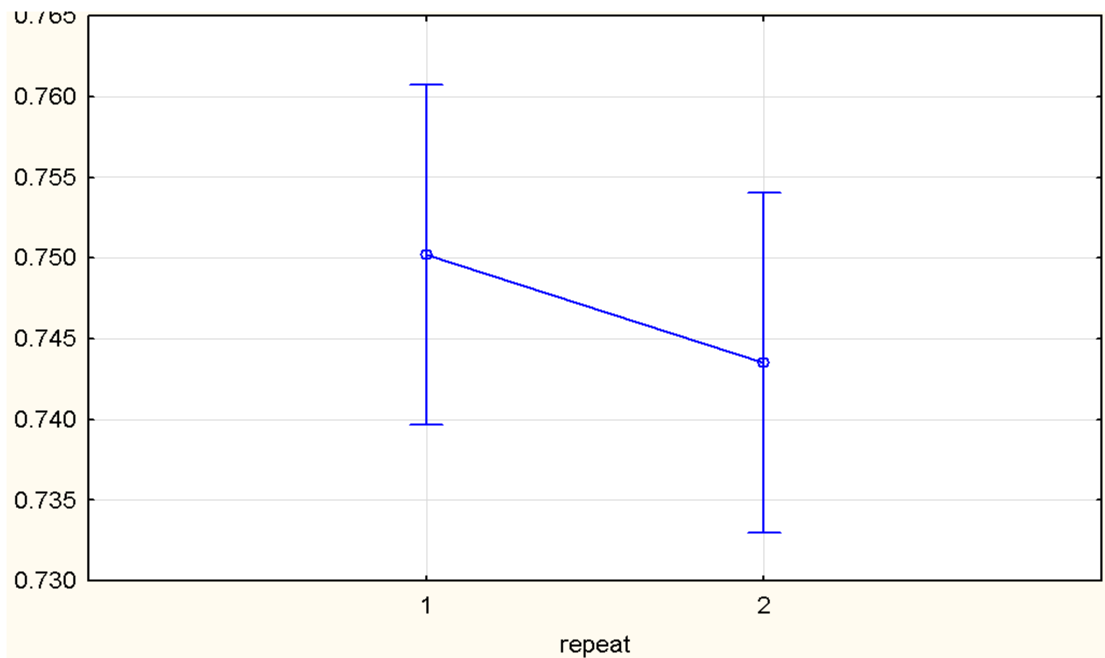


Figure 52: the output shows the two repeats were in line with each other across the serotypes at 25°C (p=0.372). The Vertical bars are overlapping and represent 95% confidence intervals.

Figure 53 Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 10°C in Nutrient media

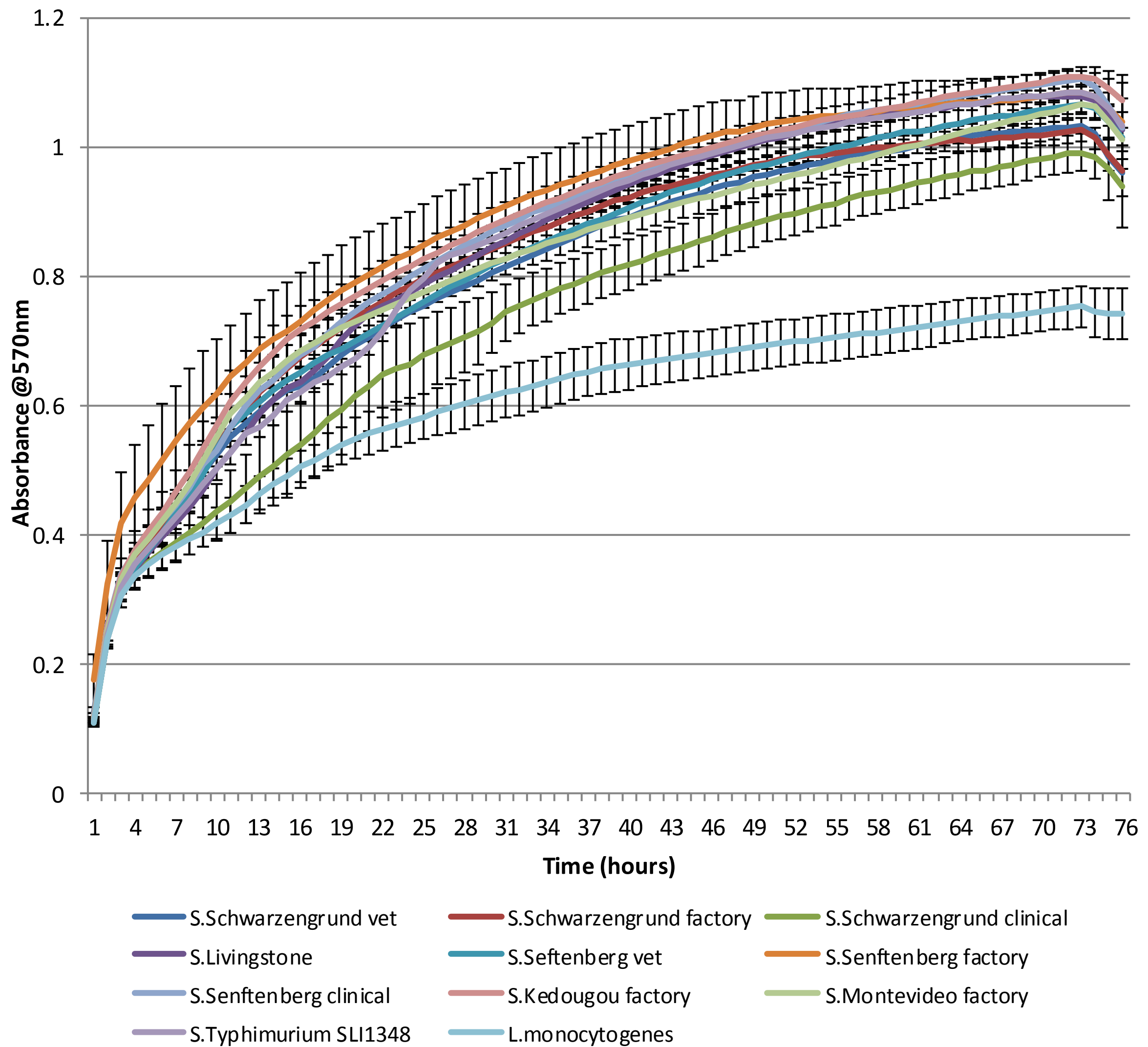


Figure 53: Automated growth curves for *Salmonella* isolates and *L.monocytogenes* 11994 at 10°C in Nutrient broth produced using the Biotek absorbance reader. This shows that all strains have the ability to grow in nutrient media with *L.monocytogenes* 11994 growing to a lower density in comparison to the *Salmonella* isolates. The clinical isolate of *S*.Schwarzengrund also seems to grow to a lower density. *Salmonella* growth ranged between 1.1 and 0.99 nm, whereas *L.monocytogenes* grew to 0.75n m. Further analysis was performed using RMANOVA to reveal any significant patterns in growth between the isolates.

Figure 54 Output from the RMANOVA to reveal the overall differences in growth of isolates in nutrient media at 10°C

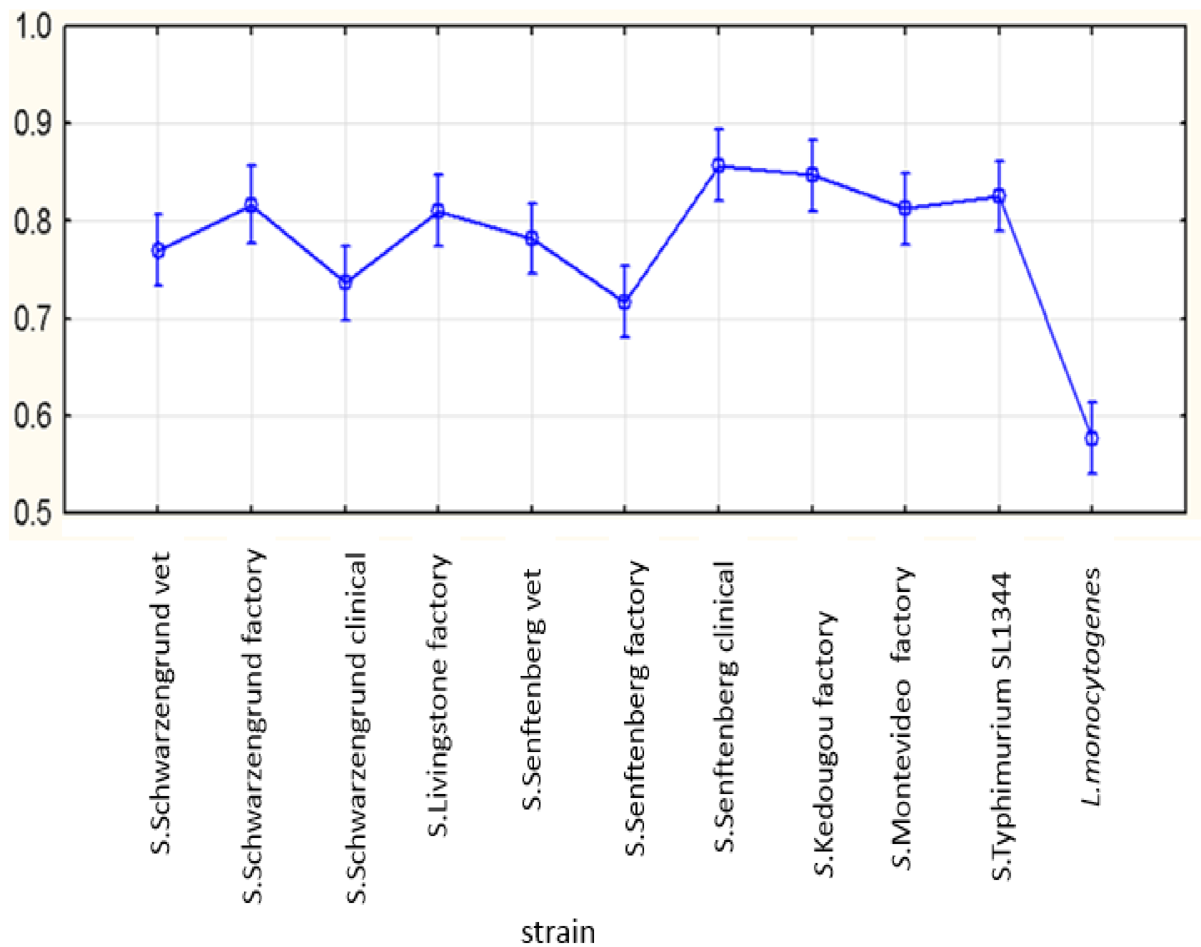


Figure 54: The Repeated measure ANOVA (RMANOVA) output shows the overall effect of growth in nutrient media on serotype at 10°C. Results indicate that there was no significant differences in the growth of factory isolates of *Salmonella* isolates however *L.monocytogenes* grew significantly slower than the *Salmonella* isolates and the growth of *S.*Senftenberg 775W was higher than serotype matched strains (p=0.000). The Vertical bars represent 95% confidence intervals.

Figure 55 Output from the RMANOVA to show the reproducibility of the growth curves in nutrient media at 10°C

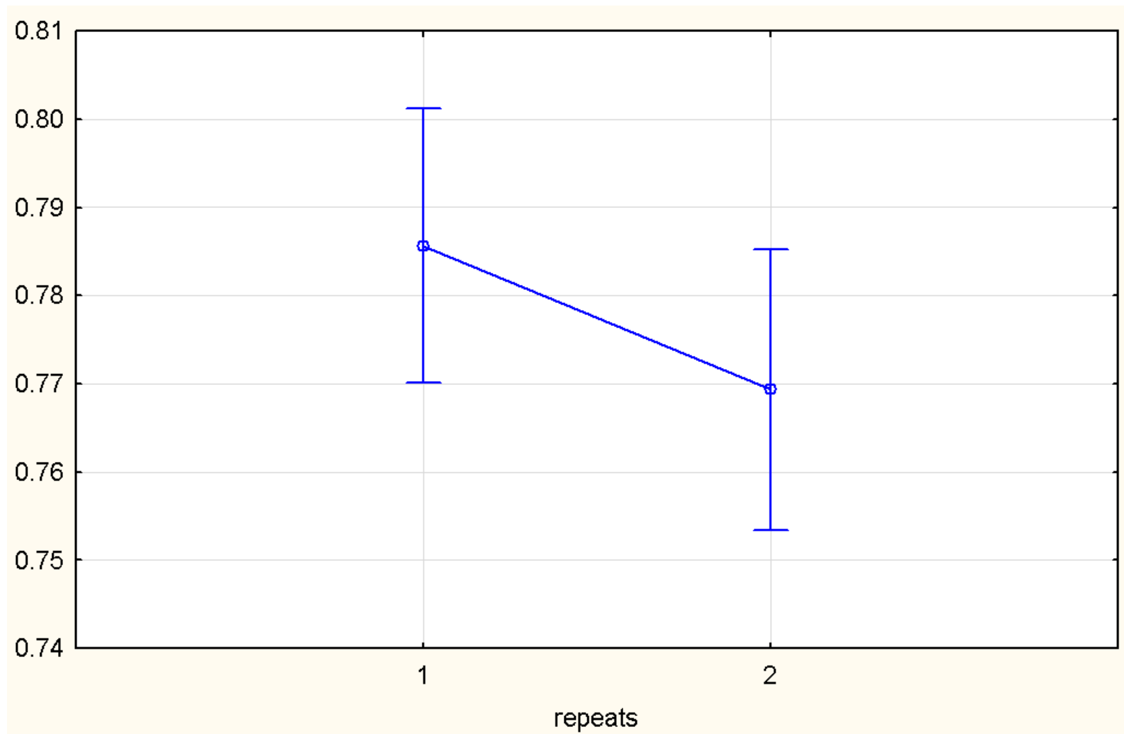


Figure 55: The output shows the two repeats were in line with each other across the serotypes at 10°C (p=0.149). The Vertical bars are overlapping and represent 95% confidence intervals

4.5 Growth in LB Broth (Luria low salt defined media)

Figure 56 Automated growth curves for *Salmonella* isolates at 37°C in Luria broth

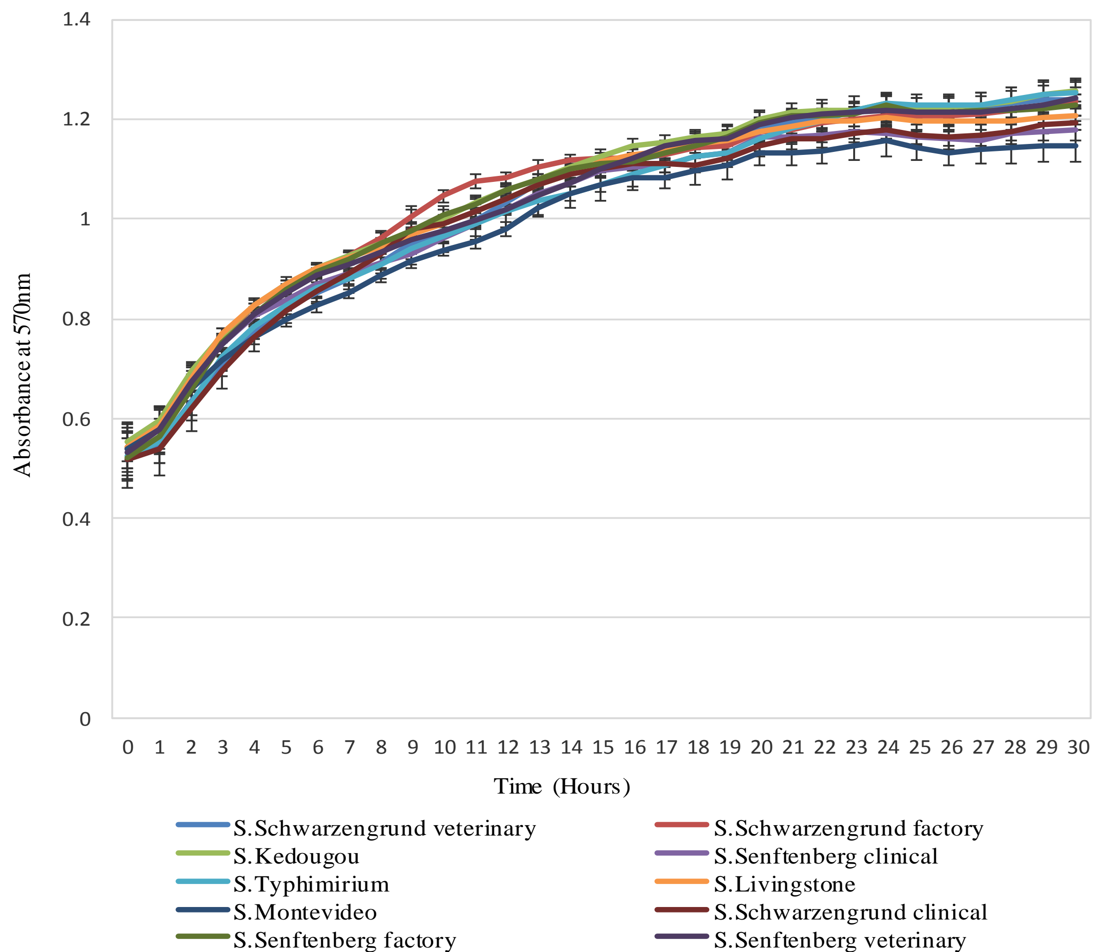


Figure 56: Automated growth curves for *Salmonella* isolates at 37°C in LB broth over 30 hours, produced using the Biotek absorbance reader. This shows that all strains have the ability to grow in LB media. The graph does not reveal any major distinctive differences in the growth between the *Salmonella* isolates, however the factory strain of *S*.Montevideo seems to have grown less in comparison to some of the isolates. Further analysis was performed using RMANOVA to reveal any significant patterns in growth between the isolates.

Figure 57 Output from the RMANOVA to reveal the overall differences in growth of isolates in Luria media at 37°C

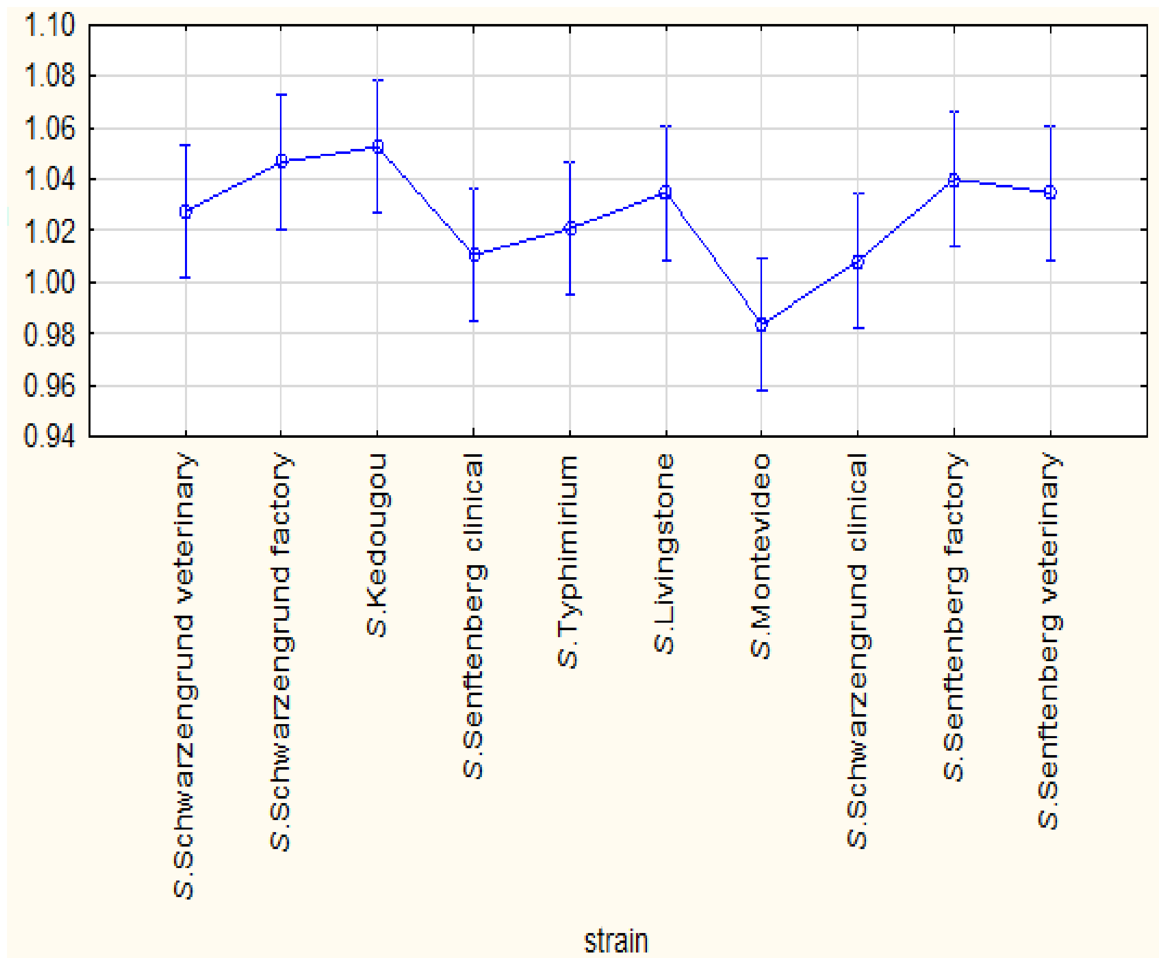


Figure 57: The Repeated measure ANOVA (RMANOVA) output shows the overall effect of growth in LB Broth at 37°C. Results indicate that there was no major differences in growth across the serotype matched isolates, however the factory isolate of *S.*Montevideo seems to have grown less in comparison to the factory isolate of *S.*Kedougou and *S.*Schwarzengrund (p=0.1876).

Figure 58 Output from the RMANOVA to show the reproducibility of the growth curves in Luria broth at 37°C

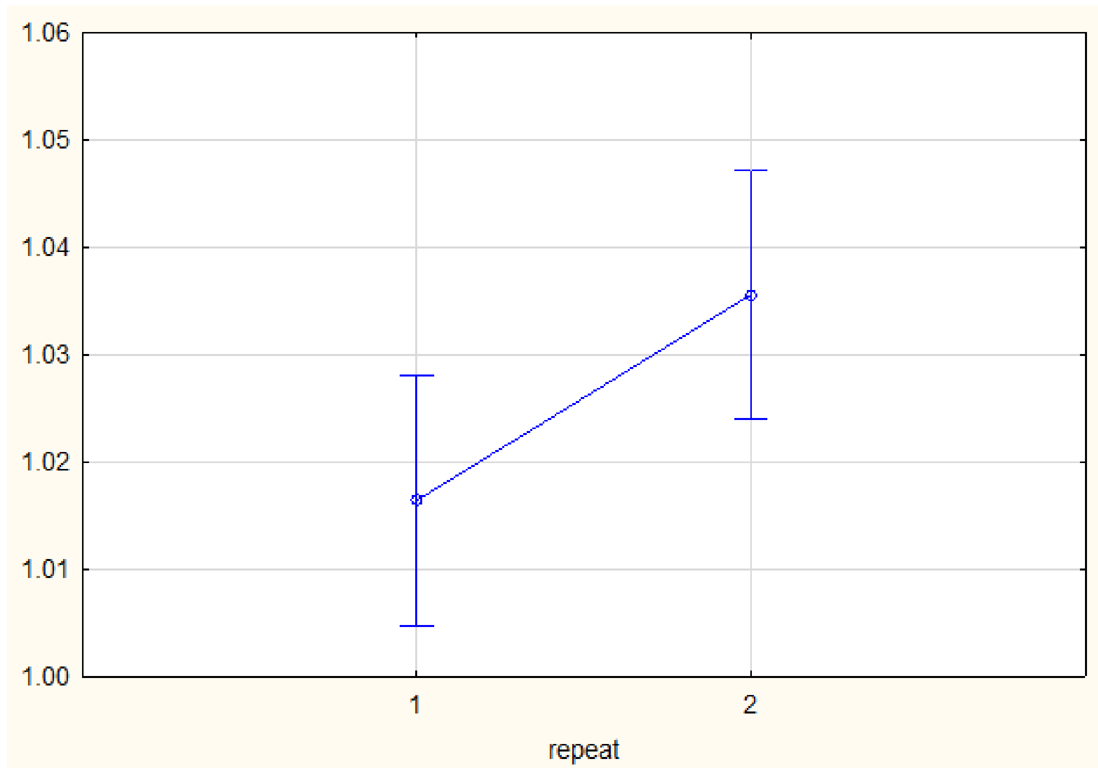


Figure 58: the RMANOVA output shows for 37°C although the first repeat was lower than the second the vertical lines representing the 95% confidence intervals are still overlapping (p=0.2315).

Figure 59 Automated growth curves for *Salmonella* isolates at 25°C in Luria broth

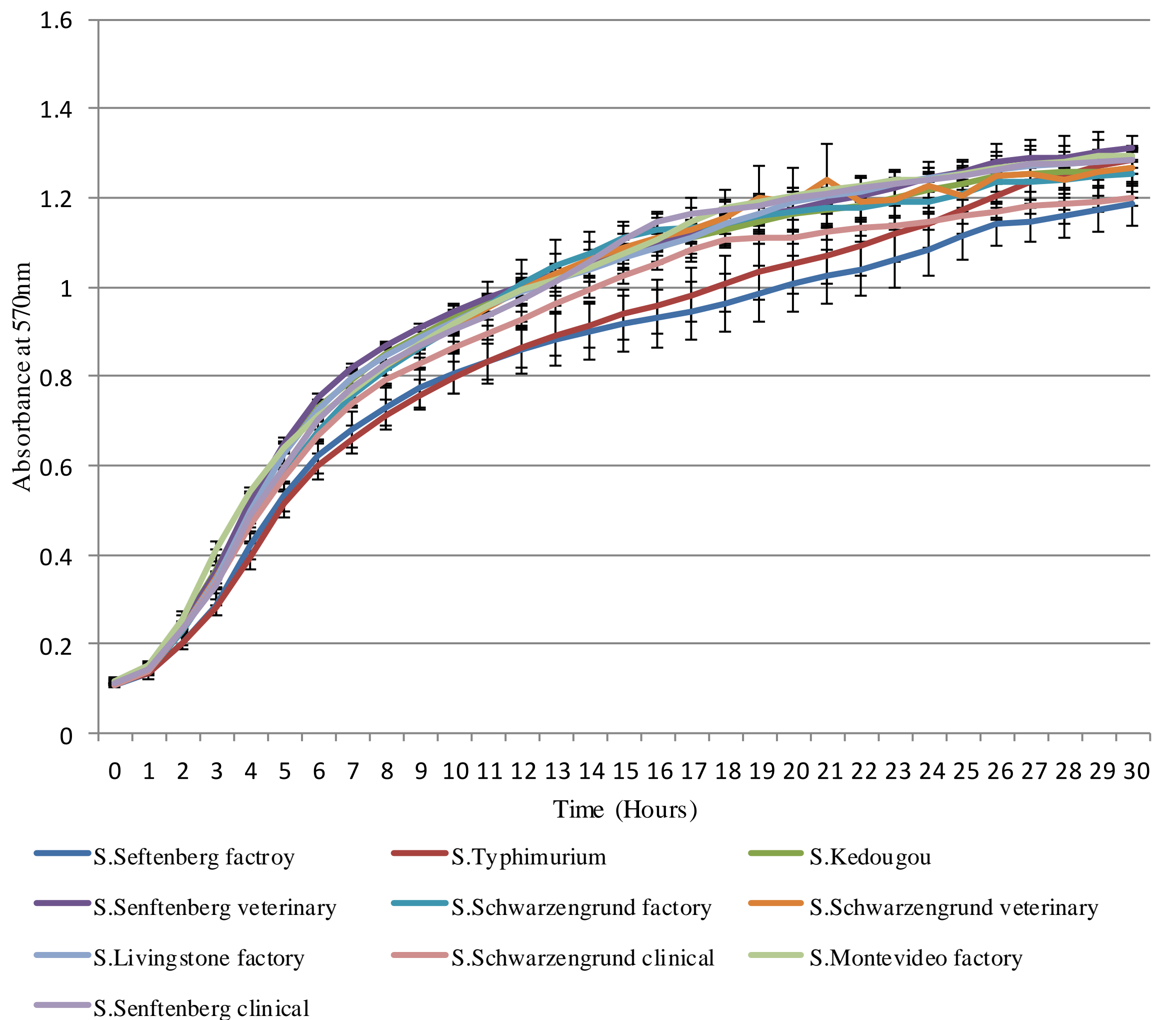


Figure 59: Automated growth curves for *Salmonella* isolates at 25°C in LB broth over 30 hours, produced using the Biotek absorbance reader. This shows that all strains have the ability to grow in LB media, some differences in growth are observed for S.Typhimurium SL1344, the factory isolate of *S*.Senftenberg and the clinical isolate of *S*.Schwarzengrund. To investigate the significance of these differences, statistical analysis was performed using RMANOVA

Figure 60 Output from the RMANOVA to reveal the overall differences in growth of isolates in Luria media at 25°C

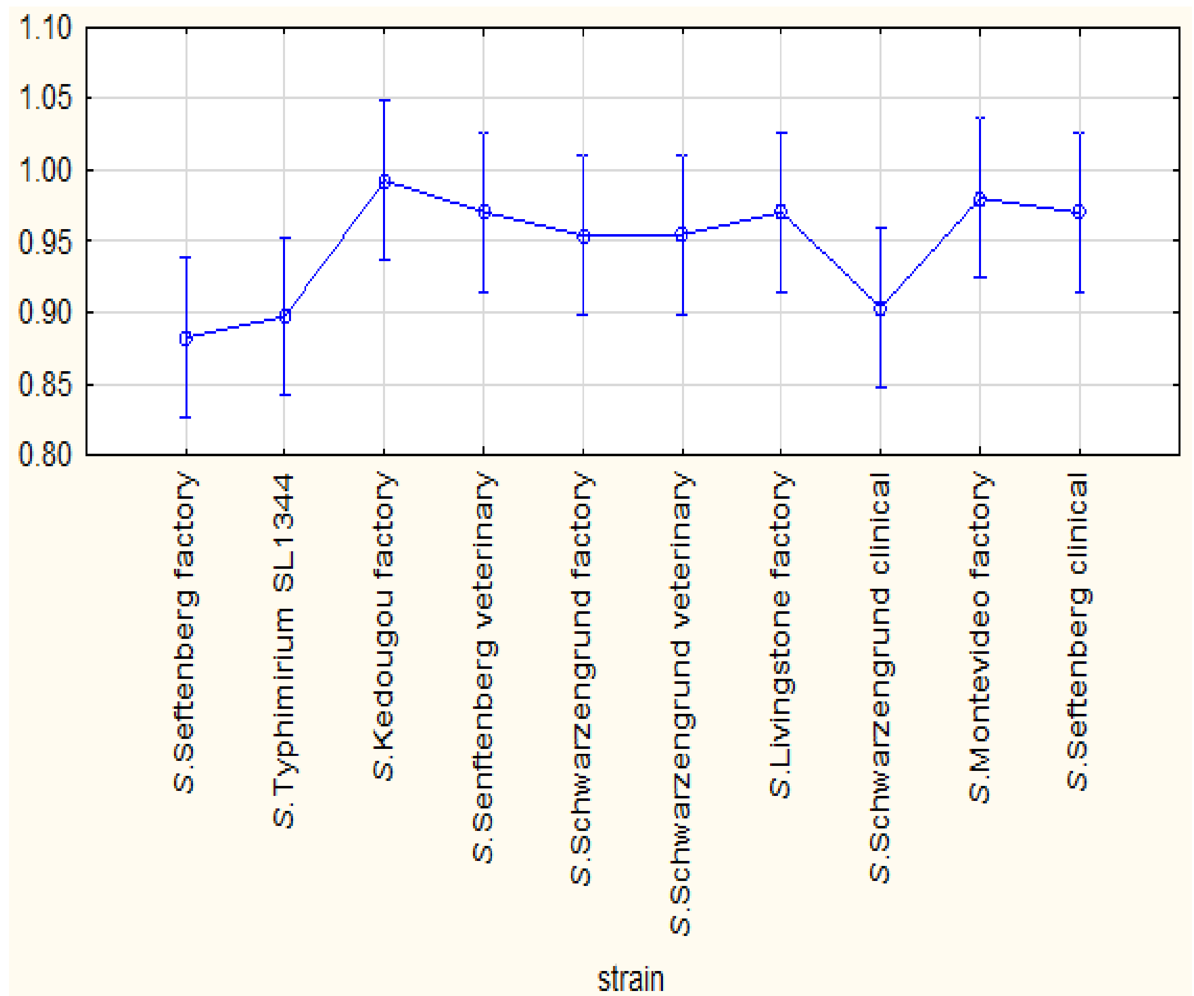


Figure 60: The Repeated measure ANOVA (RMANOVA) output shows the overall effect of growth in LB Broth at 25°C. Results show that the strains did not grow significant lower than the other isolates (p=0.069) as the vertical lines representing 95% confidence intervals are overlapping.

Figure 61 Output from the RMANOVA to show the reproducibility of the growth curves in Luria broth at 25°C

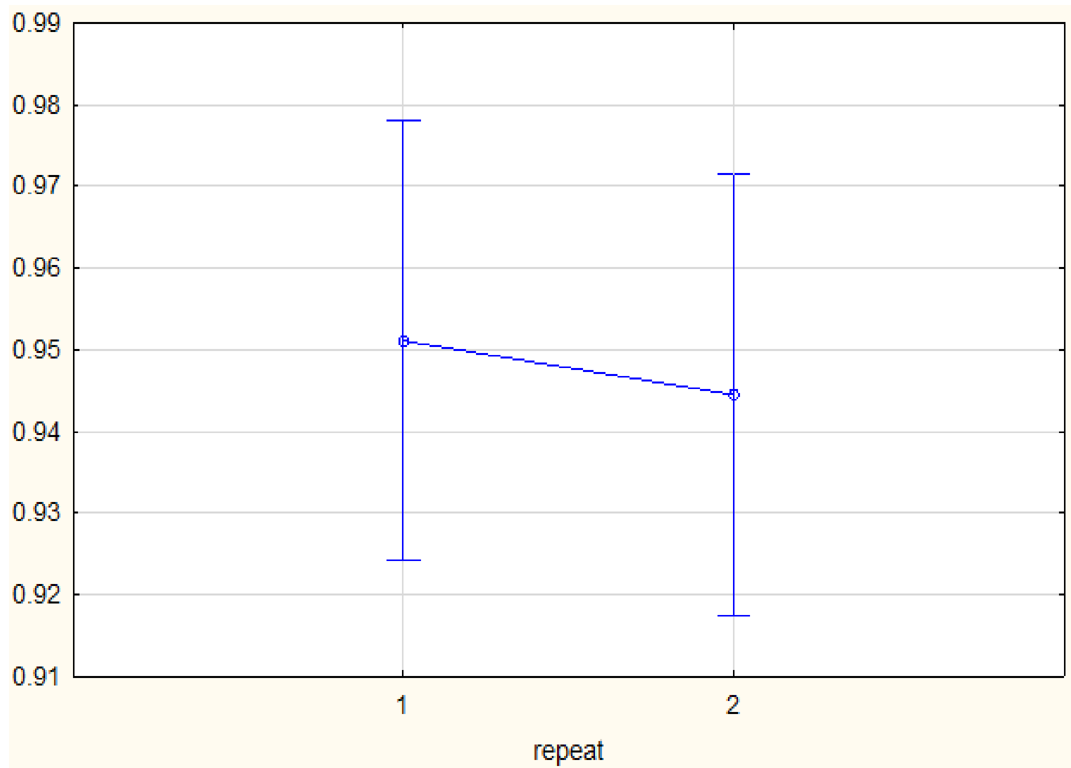


Figure 61: the RMANOVA output shows for 25°C both the repeats were in line with each other, the vertical lines representing the 95% confidence intervals are still overlapping (p=0.707).

4.6 Growth in M9 salts (minimal media)

Figure 62 Automated growth curves for *Salmonella* isolates at 37 °C in M9 salts

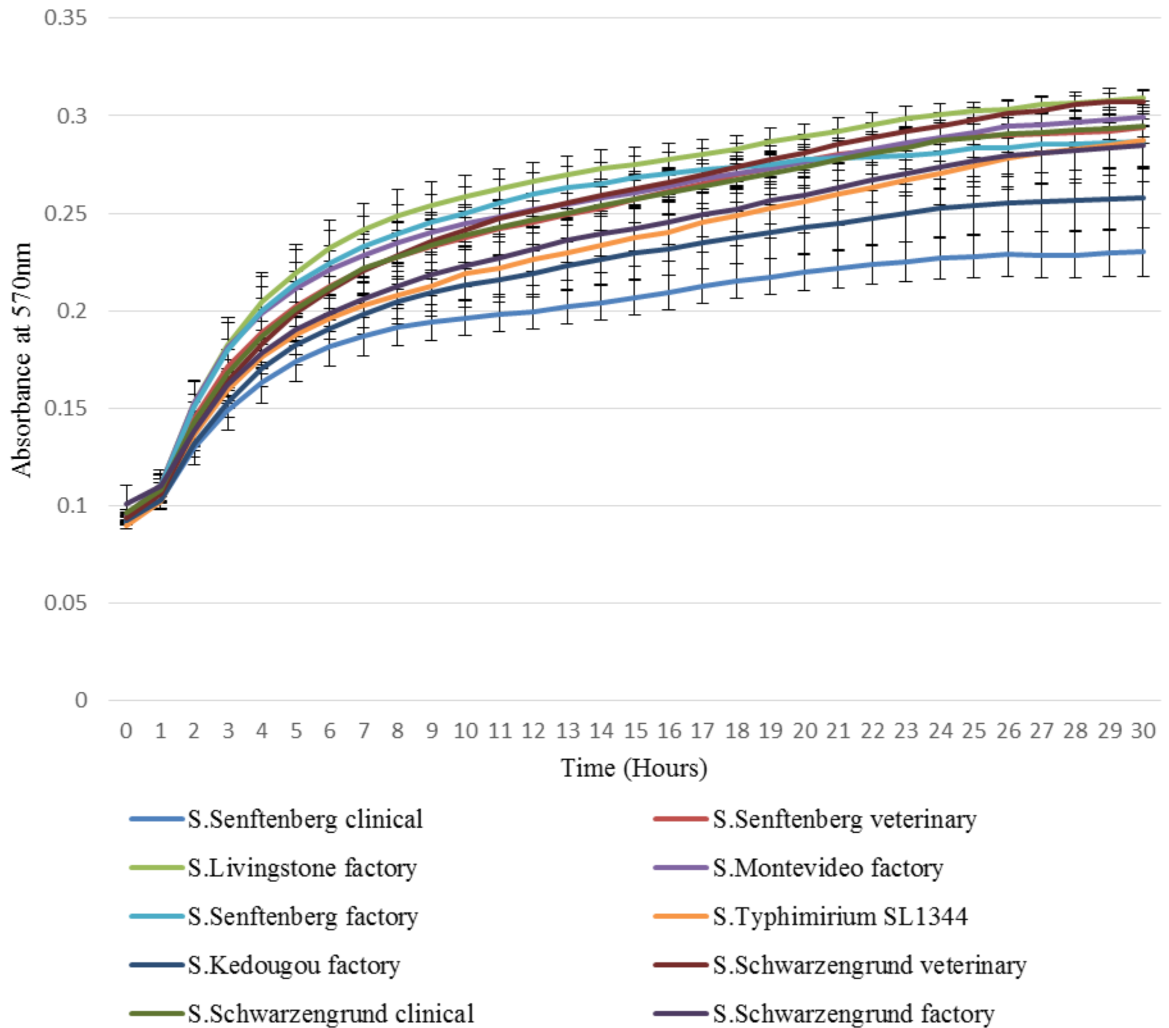


Figure 62: Automated growth curves for *Salmonella* isolates at 37°C in M9 salts with 0.6% glucose. This shows that all *Salmonella* isolates can grow in low nutrient media, although not as well as in LB Broth and Nutrient Broth. Some differences in the growth of *S*.Senftenberg 775W are observed which were investigated further using RMANOVA.

Figure 63 Output from the RMANOVA to show the reproducibility of the growth curves in minimal media at 37°C

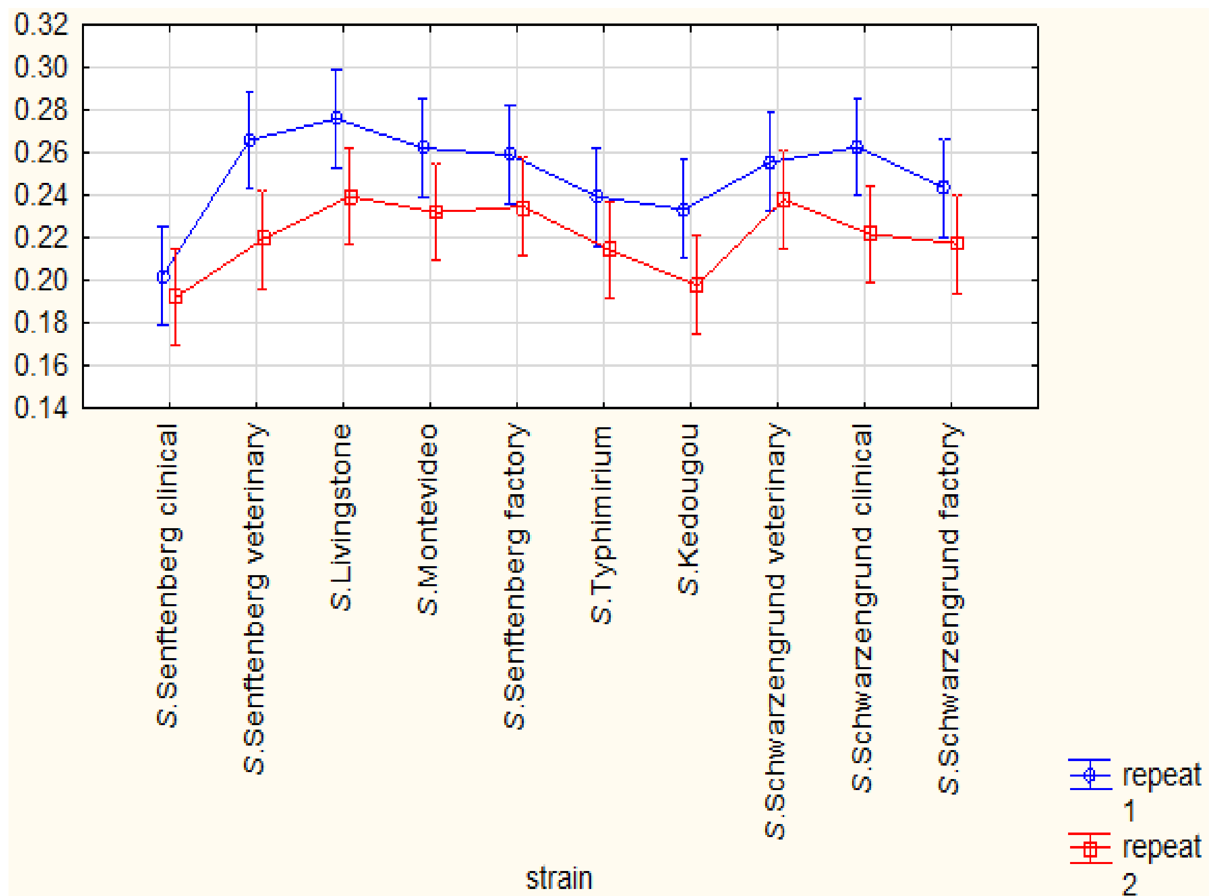


Figure 63: the output shows that although the second repeat was lower than the first, whilst there are elements of significance emerging in the first repeat, the second repeat shows the differences are not reproducible and leads to an indication that there is a diversity within the responses that is not consistent. The vertical lines represent the 95% confidence intervals (p=0.88944).

4.7 Discussion

Previous studies highlighted that contamination of factory surfaces with *Salmonella* was a growing issue (Oliveira *et al.*, 2007; Dawson *et al.*, 2007). Yet there is limited knowledge on the survival of resident factory *Salmonella* strains in comparison to other isolates. This study attempted to address this by selecting a panel of ten isolates consisting of factory, veterinary and clinical strains and comparing their survival primarily on stainless steel coupons, preliminary studies were also conducted on rusty carbon steel. The impact of temperature on survival was also investigated.

Temperature plays an important role in *Salmonella* persistence on surfaces, elevating the temperature above the optimum for bacteria can cause cell damage and death due to the cellular components being destroyed. The decimal reduction time is defined as the time at a given temperature for the surviving population to be reduced by 1 log cycle (Adams and Moss, 2000). Results have shown that on stainless steel at both 10°C and 25°C the factory strains survived better than the clinical strains and veterinary strains.

Importantly at 37°C, factory, veterinary and clinical strains are unable to survive more than 48 hours and cell death was very rapid for all serotypes, the highest D values were for the factory isolate of *S*.Montevideo D_{37}=0.283 followed by the veterinary isolate of S.Schwarzengrund D_{37}=0.295, all other isolates had a D values ranging from D_{37}= 0.13 to D_{37}=0.14. Arguably 37°C is the optimum for *Salmonella* growth, however in this study *Salmonella* showed the lowest survival on stainless steel at 37 °C. At 25°C all isolates of *Salmonella* were persistent over 22 days and the independent reading at 72 days showed a 3 to 5 log survival across the panel of isolates, with all the factory isolates exhibiting the highest D values. At 10°C the D values obtained were lower compared to 25°C but higher than those at 37°C, despite the highest D value being for the factory strain of *S*.Senftenberg (D_{10}=6.1), the remainder of the factory strains did not exhibit any increased survival in comparison to strains from the other environments.

Overall, the factory strains of *Salmonella* as a group only showed enhanced survival at 25°C compared to the other two groups of isolates, these results may be due to changes in the cell membrane of *Salmonella* at higher and lower temperatures. The normal temperature of a healthy dog ranges from 37.2 °C –39.2 °C, which is similar to the average body temperature of a human (37°C), therefore it would be assumed that clinical and veterinary isolates survive better close to body temperature and decline when environmental conditions fell from this.

However results from this study show that all strains were unable to thrive and survival was limited.

Similarly, Chaitiemwong *et al.* (2010), investigated the survival of *Listeria monocytogenes* on a conveyor belt, using identical temperatures and similar humidity levels. The study also revealed that survival was better at 25 °C in comparison to 37 °C and in line with the current study at 37 °C and 20% relative humidity; results indicated a rapid decline in survival during the first 6 hours. Moreover at 10°C and high humidity (70% RH) a rapid decrease in cell counts was also observed (Chaitiemwong *et al.*, 2010).

Salmonella is able to persist in food environments for years (Humphrey *et al.*, 1995; Nesse *et al.*, 2003). The survival of resident factory strains in comparison to clinical and veterinary strains has not been widely investigated in previous literature; results from this study indicate that all resident factory strains of *Salmonella* survived better than clinical and veterinary strains of the same serotype at 25°C and at 10°C the factory isolate of *S*.Senftenberg survived better than all the other isolates in the panel. Previously, a study was conducted by Habimana *et al.* (2010b), comparing the survival of resident flora strains in feed processing plants in response to stress factors typically found in the factory. This reported that resident flora strains (Gram-positive and Gram-negative bacteria) survived better than all the *Salmonella* isolates in humid and dry conditions over a period of 28 days (Habimana *et al.*, 2010b). They also found *Salmonella* survival was better at 30°C and 35% RH than 12°C and 85% RH with *Salmonella* levels becoming undetectable after 28 days at the lower temperature. In line with this, the current study showed survival was better on stainless steel at 25°C and 15% RH than 10°C and 70% RH and for some isolates survival was undetectable after 22 days.

Importantly, a recent study employed an identical method to investigate the survival of a panel of *Salmonella* isolates on stainless steel at 23°C and 15% RH. The panel consisted of *S*.Tenessee, *S*.Enteritidis, *S*.Napoli, *S.*Agona and several isolates of S.Typhimurium. The highest survival (4-4.5 log) was reported for S.Typhimurium DT104, *S*.Enteritidis and *S.*Agona with the average survival for *Salmonella* after 30 days being 2.5 to 3.5 logs cfu. Interestingly the study observed differences in survival within a serotype, with the highest and lowest survival being for isolates of *S.*Typhimurium (Margas *et al.*, 2013). In line with these results, the current study showed an average 3 to 5 log cfu survival was also observed after 72 days at 25°C with both factory isolates of both *S*.Schwarzengrund and *S*.Senftenberg exhibited higher D values in comparison to serotype matched clinical and veterinary isolates. Furthermore at 10°C the factory isolate of *S*.Senftenberg displayed the longest D value on steel

(D=6.1) in comparison to serotype matched clinical (D=2.1) and veterinary (D=2.02) isolates. This indicates that enhanced survival is not serotype specific.

It is thought that *Salmonella* contamination of surfaces may occur directly via food touching surfaces or indirectly due to water splashing or from footwear (Paiva *et al.*, 2009). However many methods described in literature have not included this concept in the experimental design. For example Tondo *et al.* (2010), immersed stainless steel and polythethylene coupons $20mm^2$ in size, in cultures for a range of time intervals up to 60 minutes (Tondo *et al.*, 2010). If mimicking conditions within the factory then surfaces would not be immersed in cultures for such lengthy time periods and would not be submerged to such high levels of microorganisms. In order to overcome this limitation in this study, steel coupons were inoculated with a drop containing 10μl of culture and allowed to dry- mimicking a splash.

Previous studies have concluded that *Salmonella* colonises a range of inert food contact surfaces, but at different levels of adhesion (Oliveira *et al.*, 2007). Joseph *et al.* (2001), investigated the ability of poultry isolates of *Salmonella* to form biofilms on stainless steel, plastic and cement, and found that the highest density formed on plastic, followed by cement and stainless steel. Studies comparing planktonic cells and those attached to surfaces found that cells attached to surfaces seem to be more resistant and have a raised tolerance to stress and dried cells of *Salmonella* have increased tolerance to heat (Moretro *et al.*, 2009a).

In general it is thought that the surface of stainless steel is hydrophobic (has high contact angles) and some studies have indicated that the hydrophobicity and roughness of the surface plays an important role in the adhesion and survival of bacteria (Sinde and Carballo, 2000). However Oliveira *et al.* (2007) studied the adhesion ability of four *Salmonella* Enteritidis isolates from different sources to polypropylene, polyethylene and granite. From this it was concluded that *Salmonella* adhesion could not be explained in terms of surface hydrophobicity or roughness of the materials tested but it is strongly strain dependent and the source does not affect the ability of adhesion (Oliveira *et al.*, 2007). This observation was further supported by another study which tested the adhesion ability of two poultry isolates of *S.*Enteritidis on stainless steel, results showed that adhesion for *S.* Enteritidis isolated from chicken breast was $2x10^4$ cells/mm^2 whereas adhesion for *S.*Enteritidis isolated from the water of packaged chicken was $4.67x10^3$ cells/mm^2. Therefore no correlation was made between hydrophobicity and the extent of adhesion, indicating adhesion is strain dependant (Oliveira *et al.*, 2007).

The antimicrobial activity of metals against bacterial suspensions has been widely studied, for example Faundez *et al.* (2004) found that metallic copper surfaces exhibit antibacterial activity against *S.enterica* at both 10°C and 25°C, in this study stainless steel was used as a control and showed no antimicrobial activity. Thus, the use of copper sheets in coating machinery or as surfaces in the factory may be a better alternative in decreasing *Salmonella* contamination (Faundez *et al.*, 2004).Stainless steel is usually used because of its strength, durability and it is perceived that it's an easy surface to clean and does not rust, conversely, if cleaning is not thorough or regular, pathogens may conceal in pits and scratch marks. Furthermore the surface of copper is active and can readily attack pathogens; it is thought that copper ions do this by disrupting the function of the bacterial cell membrane, interfering with the activity of proteins and causing bacterial death. In addition copper exhibits the same industrial properties as steel so can be used for buildings, machinery and other fabrics (CDA, 2012).

For years, bacterial cell death was outlined as the incapability of a cell to grow as a colony on microbiological media. The VBNC phenomenon in *Salmonella* was first suggested by Roszak *et al.* (1984) after monitoring for the presence of *S.*Enteritidis in river water, heterotrophic plate counts revealed the cells became non culturable after just 48 hours, but interestingly the addition of nutrients allowed the cells to be resuscitated (Roszak *et al.*, 1984; Waldner *et al.*, 2012). This was important as the traditional agar format was only able to detect cell growth or death and was unable to distinguish intermediate states such as cell injury. The introduction of kits employing viability indicators based on fluorescent molecules allows the evaluation at single-cell level minus cell culturing (Berney *et al.*, 2007). The LIVE/DEAD® BacLight™ Bacterial Viability Kit was used to provide a fluorescence assay of bacterial viability. It relies on the penetration of 2 nucleic acid stains which differ in their ability to penetrate cells with damaged and intact membranes, the SYTO® 9 labels cells with intact membranes a fluorescent green, whereas propidium iodide stains cells with damaged cytoplasmic membranes a fluorescent red. (Schatten and Eisenstark, 2007). The proportion of the stain combination bound to DNA varies when one stain displaces the other (Berney *et al.*, 2007). In the current study the kit was used as confirmatory test to firstly show that cells at 25°C were actually viable correlating to the cfu data and secondly to investigate whether at 37°C and 10°C when cfu data indicated organisms were no longer culturable if they were entering the viable but non culturable state. Overall the fluorescence microscopy results supported the microbiology data at both 37°C and 25°C. Initially images revealed viable and culturable

green cells as they excluded the propidium iodide, over time dead or dying cells which took up the propidium iodide were displayed as red.

Only at 10°C, when the cfu data was <10 cfu ml^{-1}, did the factory strain of *S.*Schwarzengrund completely exclude the red propidium iodide stain, indicating that the cell membrane was still intact and possibly entering the viable non culturable state. In addition to this, fluorescence microscopy images of both the clinical and veterinary isolates of *S.*Schwarzengrund revealed a proportion of green cells amongst the red cells indicating that these isolates may also be entering this state.

Other studies investigating survival of S.Typhimurium have also proposed that cells may enter a metabolically dormant phase, comparable to persistent cells found in biofilms (Apel *et al.*, 2009). Cells were stored for one month on plastic, when cfu data was <5%, baclight staining using the Live/Dead bacterial viability kit was performed which indicated that more than half of the cells were alive, although further analysis into the physiological state of these cells was not conducted, it is highly likely these cells were in the VBNC state. This implies that when used solely, traditional culturing methods were underestimating the long term survival of *Salmonella* (Apel *et al.*, 2009; Waldner *et al.*, 2012). Compared to normal culturable cells, VBNC cells have only lost the ability to grow on routine agar (Oliver, 2005; Li *et al.*, 2014), evidence suggests that the cell membrane is actually intact and contains undamaged genetic information (Heidelberg *et al.*, 1997; Cook and Bolster, 2007). Most importantly, unlike dead cells, VBNC cells are metabolically active and are able to carry out respiration, utilize nutrients and covert amino acids into proteins (Li *et al.*, 2014). One study investigating the ability of *Listeria monocytogenes* to enter the VBNC by measuring the level of ATP generated, reported elevated levels of ATP even one year after entering the VBNC state (Lindbäck *et al.*, 2010; Li *et al.*, 2014).

The VBNC state may be induced by stress or could be representing a regulated *Salmonella* survival mechanism, however as *Salmonella* is primarily a foodborne pathogen; both demonstrate a huge risk to food manufacturers and the public. The fact that cells can be resuscitated insinuates the possibility for cell growth and re-infection (Waldner *et al.*, 2012; Li *et al.*, 2014).

To survive and persist in the food factory environment it is reasonable to assume that *Salmonella* isolates might be able to grow and establish themselves more readily in nutrient depreciated conditions. Across all temperatures and media both nutritious and nutrient limited, the factory isolates of *Salmonella* did not show any competitive fitness advantage and grew in

concordance with serotype matched strains from clinical and veterinary environments.in addition, the results indicated that in minimal M9 salt media the factory isolates did also not show enhanced growth.

Nutrient broth was selected as is a commonly used non selective broth, supporting the growth of most organisms; it is often used to model any primary growth differences. Luria-Bertani (LB broth) is a nutrient-rich defined culture media which allows fast growth and yields a high cell density for a range of bacteria and it has been used to culture *Enterobactericiae* in addition to studies profiling the growth of enteric pathogens such as *E.coli* (Sezonov *et al.*, 2007). Whereas, M9 salt media are known to produce a lower cell yield as they do not contain some of the medium components in sufficient concentrations to support growth to high cell densities (Krause *et al.*, 2010). However growth in M9 salts was studied to model conditions in the food factory, it is likely isolates surviving in food manufacturing environments have a poor supply of nutrients in areas such as drains and conveyor belts. Therefore it can be assumed these isolates would grow better in media containing limited nutrients. Results showed that all the *Salmonella* isolates grew to a higher cell density in nutrient media and Luria broth in comparison to M9 salt media. The use of nutrient rich media can increase cell density and combinations of yeast extract and peptones provide the essential growth factors and vitamins cells require. Nutrient limitation can lead to cessation of growth rate and carbon starvation is associated with the induction of up to 50 bacterial proteins and resistance to bactericidal agents (Barrow *et al.*, 1996).

One study investigated the effect of biocides on factory isolates of *Salmonella* grown in nutritious TSB media and low nutrient M9 media. Although the biofilm thickness and EPS production was greater in the M9 media, it did not result in any enhanced tolerance to the biocides (Condell *et al.*, 2012). Morishige *et al.* (2014), investigated the ability of an environmental isolate of *Salmonella* Enteritidis known to enter the VBNC state, for a period of 72 hours in M9 minimal medium containing 0.8% glucose. Results indicated that the isolate was unable to retain its VBNC state in 0.8% glucose, as glucose metabolites may have led to toxicity. This was revealed via a drop in the pH of the medium to 4.7 which led to the conversion of formate to formic acid and possibly caused damage of the cell membrane (Morishige *et al.*, 2014).

4.8 Conclusion

In conclusion, the persistence of *Salmonella* on different surfaces is of great concern to the food industry as they may serve as a focus for cross- contamination of food product. The results from this study indicated that *Salmonell*a survival on stainless steel is affected by environmental temperatures that may be experienced in a food processing environment; with higher survival rates at temperatures close to 25°C and lower humidity levels of 15% RH, however a rapid decline in cell count with lower temperatures of 10°C and higher humidity of 70% RH. Also several resident factories strains survived in higher numbers on stainless steel compared to serotype matched clinical and veterinary isolates. Factory isolates of *Salmonella* did not show an enhanced growth rate in comparison to serotype matched isolates grown in luria broth, nutrient broth and minimal media, indicating that growth is unlikely to be a major factor driving *Salmonella* persistence. The fluorescence microscopy images of samples stained with BacLight supported the microbiology data at 37°C, 25°C and 10°C, however at 10°C when the cfu data was <0, results revealed that the factory, clinical and veterinary isolates of *S*.Schwarzengrund were excluding propidium iodide and possibly entering the VBNC state. However, undertaking further investigations to understand the interplay between temperature and humidity levels and modelling conditions in the food factory environment to identify areas where *Salmonella* can harbour, is key in eliminating the organism from food processing environments.

5 Chapter 5 Investigating the biofilm formation capability of isolates in the challenge panel

Introduction

Microorganisms are capable of surviving in nutrient depleted conditions and an important factor enabling them to do so is their ability to form biofilms (Srey *et al.*, 2013; Seixas *et al.*, 2014). A biofilm is classified as a population of microbial cells that are irreversibly (not detached by gentle washing) associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan and Costerton, 2002). The cells in a biofilm produce proteinaceous substances which allow protection from environmental stresses. Dutch scientist Van Leeuwenhoek in 1683 was first to show that the sticky mass of dental plaque consisted of tiny bacteria. He also found that only the surface layers of bacteria were vulnerable however the deeper layers resisted vinegar (Meyer-Lueckel and Paris, 2013). In 1978 this idea was further developed and findings showed that bacteria in a biofilm grew in a matrix which allowed them to bond tightly to a surface and perform differently from planktonic cells (Costerton *et al.*, 1995b). Biofilms can form on both abiotic and biotic surfaces and studies have shown that biofilms constitute 80% of microbial infections in the body as they are often resistant to antibiotics making them a primary health concern (Romero *et al.*, 2008). The ability of microbial pathogens to adhere to a surface, to form as a community and produce extracellular polysaccharides (EPS) is what allows them to successfully form biofilms (Vu *et al.*, 2009).

5.1.1 Stages of biofilm formation

The five major steps involved in biofilm formation can be seen in Figure 65. The initial step involves the reversible attachment of planktonic cells to a solid surface. Extracellular organelles and proteins including flagella, Curli fibers, pilli, and outer membrane proteins are involved in sensing and attaching to surfaces. The second step consists of the irreversible attachment of the cells to the surface. The adhesion between cells and surfaces is facilitated by the secretion of an extracellular polymeric substance (EPS) containing DNA, lipids, proteins and lipopolysaccharides. In the third step, the cells attached to the surface replicate and grow into microcolonies. The secretion of EPS allows the bacteria to be encapsulated in a coat of the hydrogel, acting as physical barrier amid the bacterial community and the extracellular environment. The fourth step involves the community of cells growing into a three-dimensional structure and maturing into a biofilm as cells replicate and the EPS build

up. The cells in a biofilm are tightly arranged by the EPS, allowing biofilms to show resistance to mechanical stresses preventing detachment of the biofilm from surfaces from the surfaces and chemicals stresses such as biocides. In the ultimate step, some cells separate from parts of the biofilm and promote dissemination in the environment, here they may return to their planktonic state or also have the potential to attach to other surfaces and form biofilms in other environments (Kumar and Anand, 1998; Renner and Weibel, 2011).

Figure 64 A graphical representation of the five stages of biofilm formation

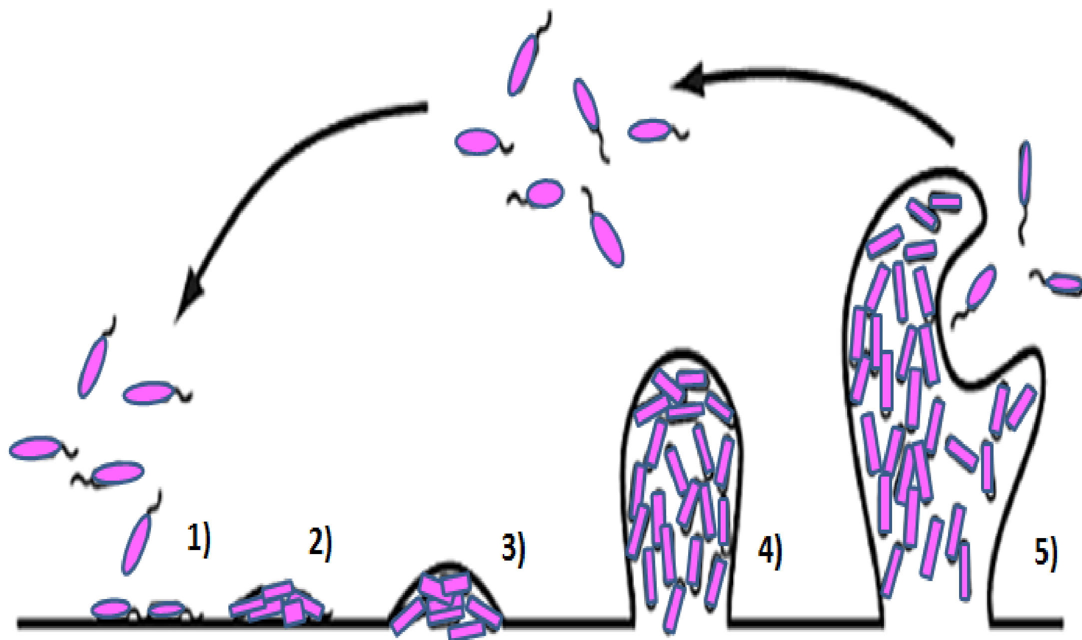


Figure 64: the five stages of biofilm formation 1) initial attachment 2) irreversible attachment 3) maturation (i) 4) maturation (ii) and 5) dispersion

Outbreaks associated with food products highlight the growing need to investigate the source of *Salmonella* contamination and identify how *Salmonella* is able to persist in the factory environment. Microorganisms are able to adhere to food processing surfaces and in the food industry this is clearly problematic. Studies have shown that the persistence of *Salmonella* is correlated to its ability to form biofilms (Habimana *et al.*, 2010b; Kostaki *et al.*, 2012). Biofilm formation can occur almost universally where microorganisms and surfaces are in contact (Kostaki *et al.*, 2012) as they can attach themselves to both living and inert surfaces (Vu *et al.*, 2009; Habimana *et al.*, 2010a). Laboratory studies have shown that a biofilm formation relies on the availability of nutrients, pH, temperature, cell structures such as flagella and ionic concentration. The ability of bacteria to adhere to food contact surfaces and

produce biofilms depends on various factors, such as the physicochemical properties of the surface of bacterial cells, the hydrophobicity and roughness of the surfaces in the factory (Oliveira *et al.*, 2007).

5.1.2 Biofilms in the food industry

The ability of bacteria to form biofilms has sparked research interest for many organisations from the food safety management perspective (Vestby *et al.*, 2009b; Vestby *et al.*, 2009a; Shi and Zhu, 2009), especially as biofilms are difficult to control as they may form in areas of factory plants where cleaning is difficult (Djordjevic *et al.*, 2002). Biofilms have serious implications on the food industry as the detachment of cells in a biofilm can lead to cross contamination of food products, causing spoilage as well as the transmission of infectious disease. Both *Salmonella spp.* and *Listeria monocytogenes* are imperative pathogenic bacteria, which can constitute biofilms on a range of surfaces such as; plastic waste water pipes (Hurrell *et al.*, 2009; Kostaki *et al.*, 2012), glass (Prouty and Gunn, 2003), rubber conveyor belts (Arnold and Silvers, 2000), cement to represent concrete floors (Joseph *et al.*, 2001) and stainless steel (Stepanovic *et al.*, 2004; Habimana *et al.*, 2010b; Kostaki *et al.*, 2012). The use of plastic materials is becoming increasingly popular for the construction of pipework, surfaces and accessories and experiments investigating biofilm formation capabilities have been conducted in plastic micro titre plates (Stepanovic *et al.*, 2004). The attachment of biofilms to surfaces is thought to be principal for the survival and persistence of both pathogens in food manufacturing environments, evidence suggests some strains can survive on the surface of equipment for many years (Lunden *et al.*, 2000; Moretro and Langsrud, 2004; Vestby *et al.*, 2009a; Kostaki *et al.*, 2012). These persistent niches where *Salmonella* can form biofilms can be a focus of cross contamination for the food environment as well as food products.

Having a better understanding of the parameters affecting biofilm formation on surfaces like plastic and steel, as well as investigating the ability of the food factory isolates to form biofilms in comparison to serotyped matched isolates from other environments (such as those isolated from the clinical and veterinary setting), could provide the information necessary to control and prevent biofilm formation thus reducing subsequent foodborne outbreaks.

Therefore, the aim of this chapter was to establish the biofilm formation capacity of the panel of *Salmonella* and *Listeria monocytogenes* isolates at different temperatures and times in both nutrient rich and nutrient deprived media. Specific objectives included;

- Identifying whether all the strains in the selected were capable of forming biofilms at 24 hours in a 96 well micro titre well plate format and whether enhanced biofilm formation was achieved through increased incubation of 48 hours.
- Investigating the effect of temperature by incubating plates at 37°C, 25°C, 15°C and 10°C.
- Investigating the effect of media/nutrients on biofilm formation using full strength TSB and 1/20 diluted TSB media.

5.2 Material & Methods

5.2.1 Bacterial strains used in study:

The study panel as described in Table 3 of Chapter 2 was used to study biofilm formation.

5.2.2 Media and equipment

Tryptone soya broth (TSB) and 1/20 Tryptone soya broth were used as a growth medium, and was purchased from Oxoid (Basingstoke, U.K.), prepared according to manufacturer's instructions and sterilised at 121 °C for 15 minutes and stored at 4 °C until required. The 1/20 TSB was made by adding 6 grams to 1 litre of SDW water and autoclaved at 121°C for 15 minutes.

Sterile polystyrene (Fisher Scientific, UK) 96 flat well plates were used to grow biofilms. An 8 multi-channel pipette (XL 3000i) (Denville Scientific) was used in the study which had a range from 20 – 200 µl. The optical density of the wells was measured in the BIOTEK Elx808 Absorbance micro plate reader and this provides the flexibility to manipulate and analyse data.

The absorbance data from the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ data were exported from the plate reader software to a Microsoft Excel spreadsheet. The data were then exported into STATISTICA (data analysis software system), version 10, (USA) and analysed by selecting the Factorial ANOVA tool. This allows comparison of the multiple factors that may affect biofilm formation across the panel of strains.

5.2.3 Biofilm formation method

The method followed in this study was adopted from a study conducted by Stepanovic *et al.* (2004). A bead from each strain was added to a universal tube containing 20ml of TSB media and incubated overnight at 37°C. The following day 5 ml of the overnight inoculum was added to 5 ml of neat TSB in a sterile universal tube and vortexed for 60 seconds. The Optical Density (OD) was noted at 600nm and dilutions were performed as required to ensure a concentration of 10^6 CFU were added to each well. To investigate the effect of full strength medium, a 230µl volume of neat TSB was added to the wells, in the same plate 230µl of 1/20 TSB was added to show the effect of diluted medium, each experiment had 2 technical repeats. A 20µl volume of diluted o/c was added to each well of both rows and incubated for 24 hours and 48 hours respectively. The experimental procedure was repeated four times. After 24 hrs the medium was changed for the 48 hour plates which were then re-incubated. For the 24 hour plates, medium was removed with a pipette and each well was washed with 300µl of SDW water twice. A 250µl volume of methanol (Fisher Scientific) was added to each well to fix the bacteria and left for 15 minutes before being poured off. The plates were air dried. Then a 250µl volume of crystal violet dye was added to each well and left for 10 minutes and then poured off and the remaining stain was removed by washing under tap water, the plates were again air dried. Finally a 250µl volume of 33% glacial acetic acid was added to each well which re-dissolved any bacteria and the plates were left at room temperature for 30 minutes. The optical density of each well inoculated was measured with BIOTEK Elx808 Absorbance micro plate reader (Biotek, UK) at 570nm and results were imported into an Excel spreadsheet (Microsoft, 2010). This method was repeated for the investigation of biofilm formation at 37°C, 25°C, 15°C and 10°C.

5.3 Results

5.3.1 Biofilm formation at 37°C

The results at 37°C as indicated in Figure 66 revealed that all of the strains in the panel were capable of forming biofilms. The output from the Factorial ANOVA shown in Figure 65 demonstrated that *S.*Senftenberg 775W was the strongest biofilm producer compared to all other strains. Overall there was a difference in biofilm formation between the remaining strains however it was not statistically significant (p=<0.05). The results as shown in Figure 66 also revealed that using 1/20 TSB medium compared to full strength TSB medium showed no significant increase in biofilm formation. All of the strains showed higher levels of biofilm formation with 48hours incubation compared to 24hours as shown in Figure 67. A Post Hoc test as shown in Table 8 was conducted to analyse the effect of time, media and serotype in addition to the interactive effect of the three parameters. Overall no factory strains demonstrated an enhanced ability to produce biofilms in comparison to matched clinical and veterinary strains.

Table 8 Post Hoc test at 37°C to reveal the effect of time, media and serotype on biofilm formation

Effect	Wilks Value	F	Effect df	Error df	p
Time	0.794	4.049	8	125	.000*
Media	0.881	2.114	8	125	.0485
Serotype	0.044	6.378	80	801.4	0.000*
Time*media	0.86	2.55	8	125	.013*
Time*serotype	0.469	1.271	80	801.4	0.062
media*serotype	0.501	1.155	80	801.4	0.176
Time*media*serotype	0.431	1.421	80	801.4	0.12*

Table 8: A summary of the overall effect of time, media and serotype on biofilm formation at 37°C. The p values in the table indicate there was no significant difference between full strength and 1/20 TSB media on biofilm formation (p=0.485), there was a significant difference between the 24 hours and 48 hours incubation time (p=0.000) and across serotypes (p=0.000). However there was no combined difference between media and serotype (p=0.176) and incubation time on serotype (p=0.062). These results were explored further in the factorial ANOVA outputs.

Figure 65 Summary output produced using Factorial ANOVA highlighting differences in biofilm formation across strains at 37°C

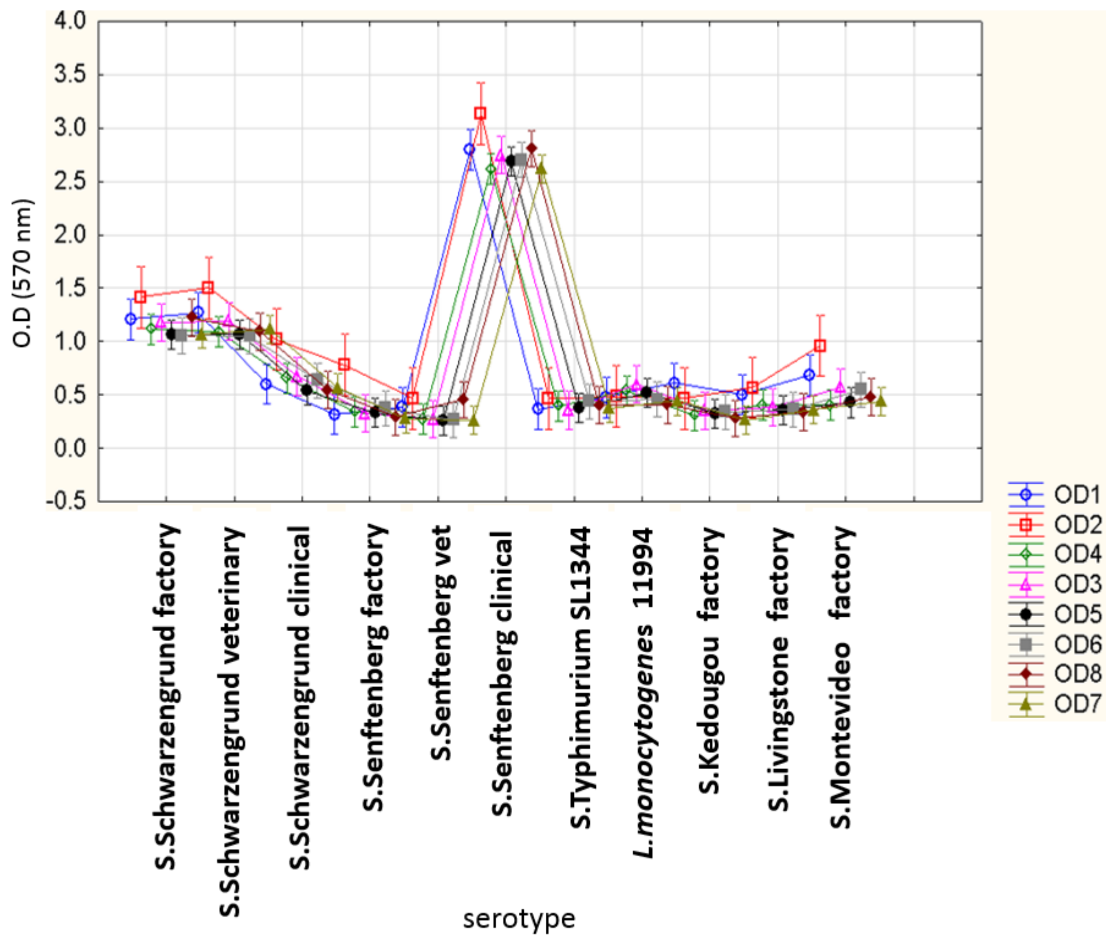


Figure 65: The factorial ANOVA output displays the mean biofilm formation for each strain and shows the overall effect of serotype and time at 37°C. Importantly, results indicated that all the strains in the panel could form biofilms and *S.* Senftenberg 775W was the strongest biofilm producer (p=0.000), followed by the factory isolate of *S.*Schwarzengrund and the veterinary isolate of *S.* Schwarzengrund. The Vertical bars represent 95% confidence intervals

The media concentration is a known factor that influences biofilm concentration therefore the ability of isolates to form biofilms in full strength TSB media and diluted 1/20 TSB media was investigated as shown below.

Figure 66 Effect of full strength and diluted TSB media on biofilm formation at 37°C

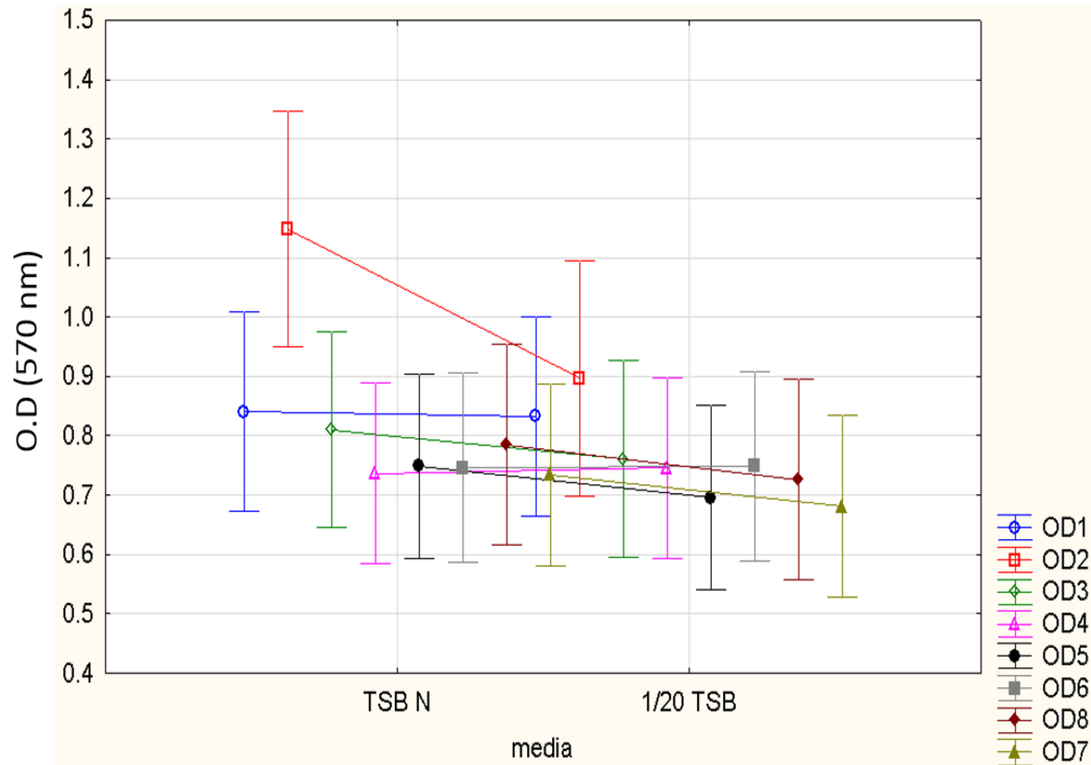


Figure 66: Biofilm formation in TSB and diluted 1/20 TSB media at 37°C: based on eight observations there is no significant difference in biofilm formation in TSB and the diluted 1/20 TSB media (p=0.4845). Vertical bars represent 95% confidence intervals.

Increased incubation time is a known factor that influences biofilm production; this was explored in the factorial ANOVA output below.

Figure 67 The effect of time on biofilm production at 37°C

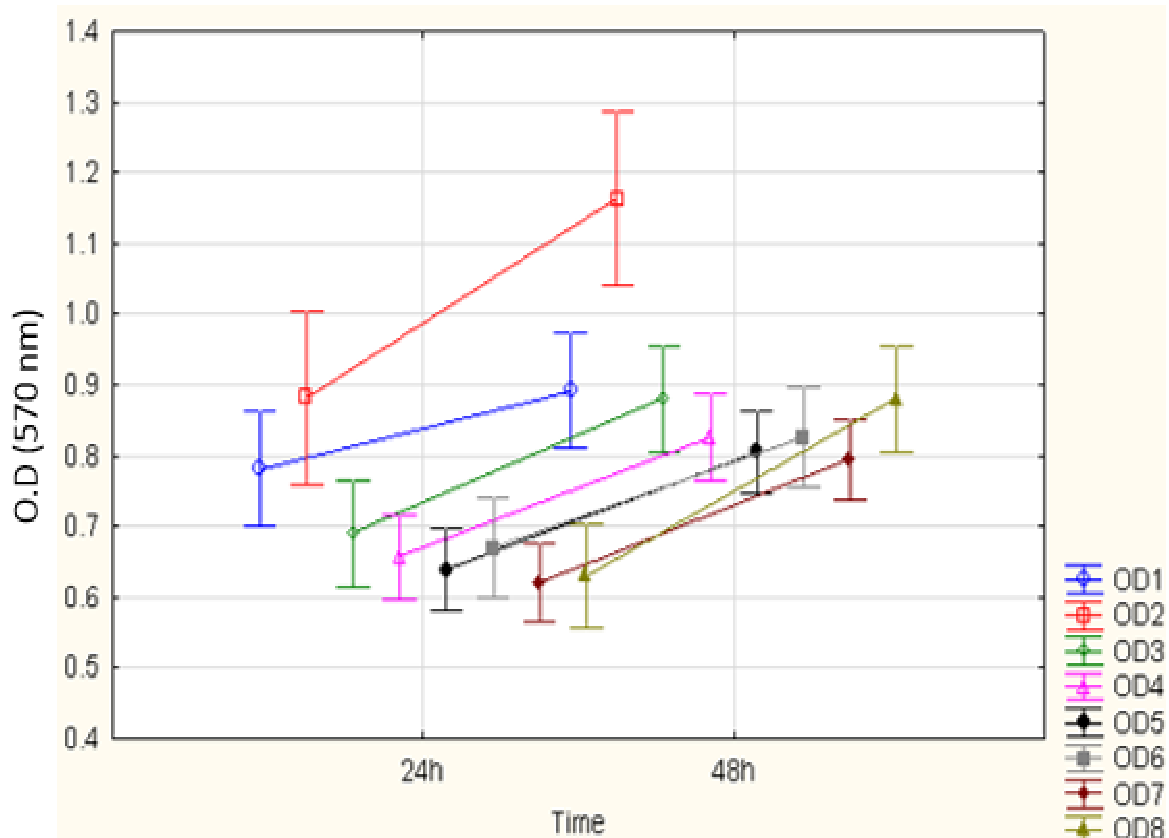


Figure 67: Effect of time on biofilm formation at 37°C: Overall the trend from the graph indicates increased biofilm formation with 48 hour incubation compared to 24 hours incubation. Of the eight observations, OD2 to OD7 show a potential significant difference of time (p=0.00042); however OD1 shows no significant difference. The vertical bars represent 95% confidence intervals.

5.3.2 Biofilm Formation at 25°C:

The results of biofilm production at 25°C as shown in Table 9 revealed a similar trend to biofilm production at 37°C, with *S.* Senftenberg 775W being the strongest biofilm former, followed by the factory and veterinary strains of *S.* Schwarzengrund All the strains produced significantly higher biofilms in diluted 1/20 TSB medium compared to full strength TSB medium (p=0.000). Time also played a role in biofilm production at 25°C, revealing higher production after 48hours incubation (p=0.000).

Table 9 Post Hoc test at 25°C to reveal the effect of time, media and serotype on biofilm formation

Effect	Wilks Value	F	Effect df	Error df	p
Time	0.632	9.079	8	125	.000*
Media	0.192	2.973	80	801.4	.000*
Serotype	0.764	4.835	8	125	.000*
Time*media	0.455	1.325	80	801.4	.035*
Time*serotype	0.934	1.102	8	125	0.366
media*serotype	0.433	1.414	80	801.4	0.013*
Time*media*serotype	0.486	1.208	80	801.4	0.112

Table 9: a summary of the overall effect of time, media and serotype on biofilm formation at 25°C: The p values in the table indicate that time, serotype and media all had a statistically significant effect on biofilm production (p=0.000). The combinations of incubation time and media strength (0.035) in addition to media strength and serotype (p=0.0.13), showed biofilm production also increased significantly. However the combination of time and serotype showed no enhanced effect on biofilm formation (p=0.366). These parameters were explored further in the factorial ANOVA outputs below.

Figure 68 Summary output produced using Factorial ANOVA highlighting differences in biofilm formation across strains at 25°C

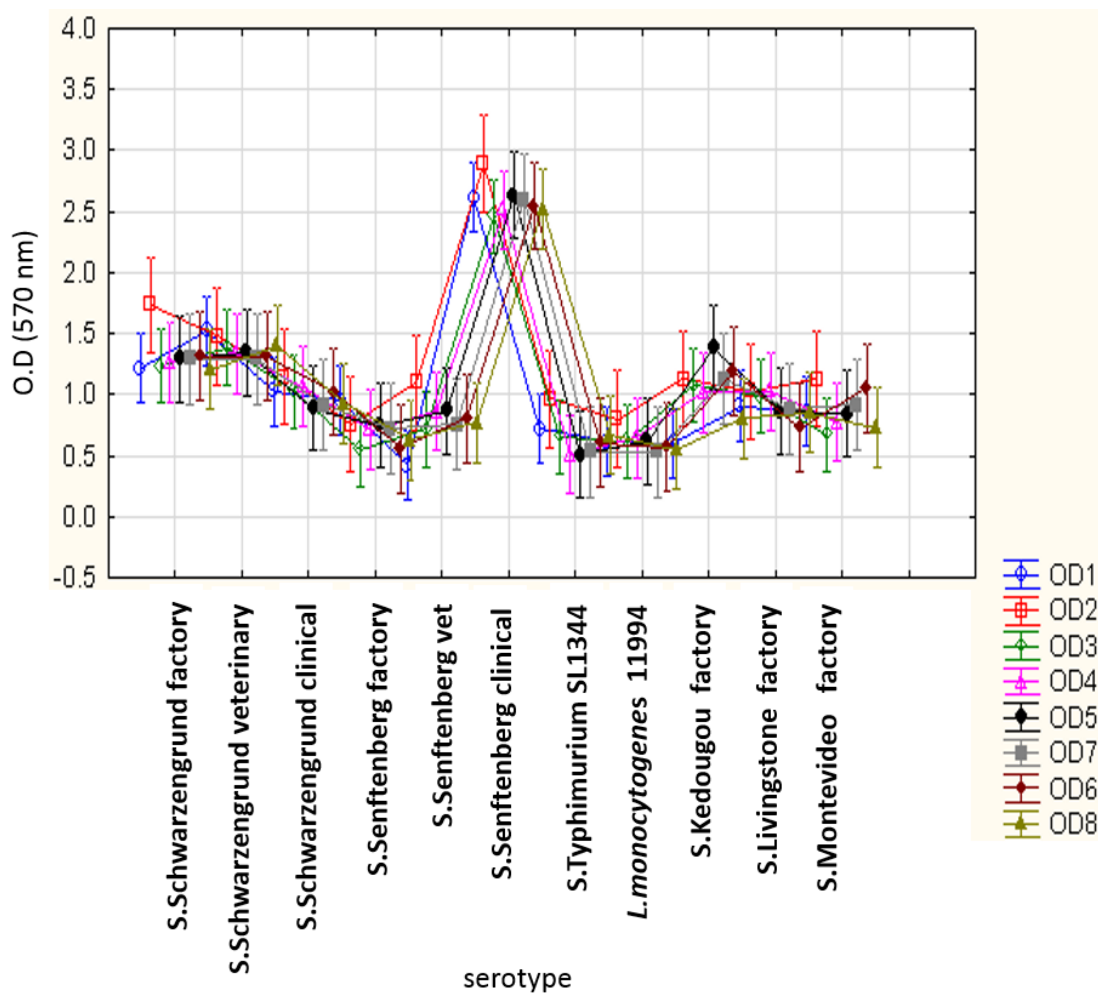


Figure 68 The factorial ANOVA output displays the mean biofilm formation for each strain and shows the overall effect of serotype and time at 25°C. Importantly, results indicated that all the strains in the panel could form biofilms and *S.* Senftenberg 775W was the strongest biofilm producer (p=0.000), followed by the factory isolate of *S.*Schwarzengrund and the veterinary isolate of *S*. Schwarzengrund. The vertical bars represent 95% confidence intervals.

Figure 69 The effect of time on biofilm production at 25°C

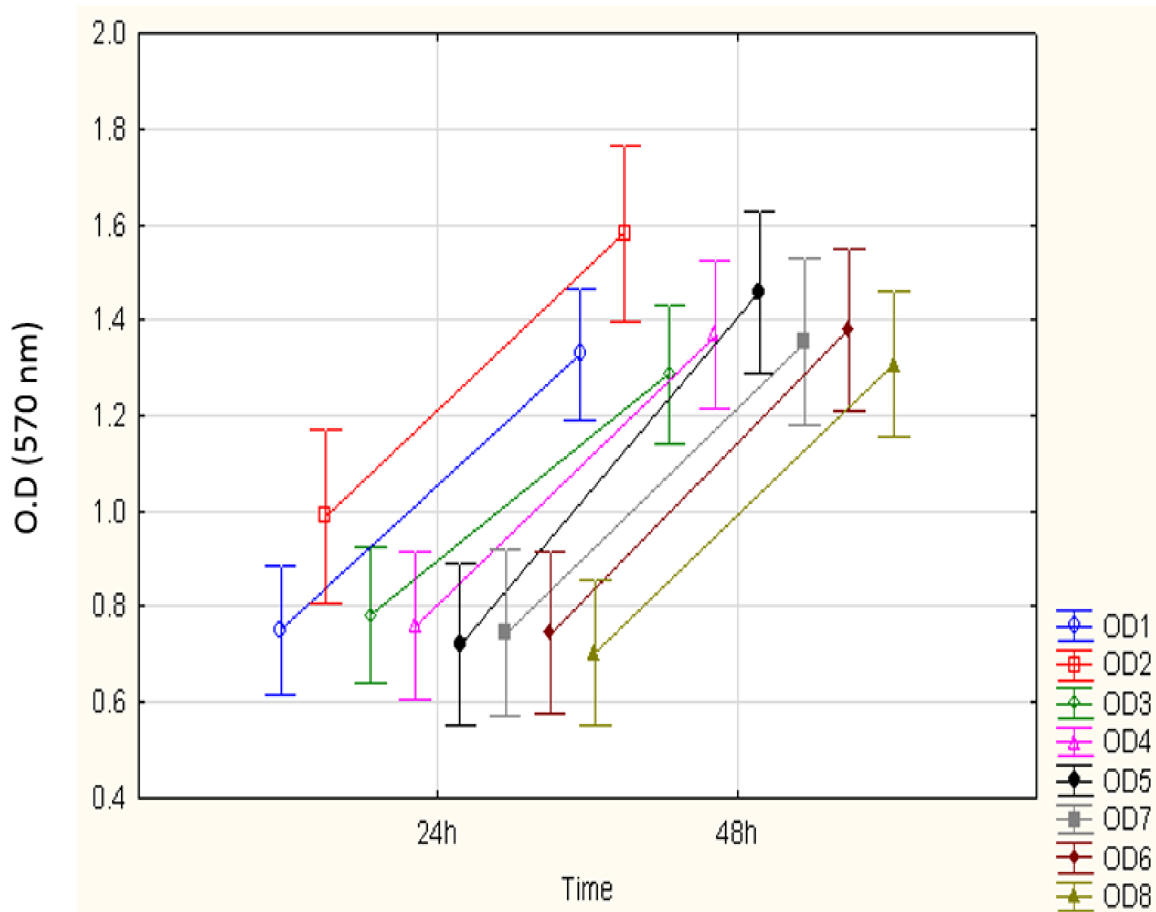


Figure 69: Effect of time on biofilm formation at 25°C. The results from eight observations indicated that biofilm formation was significantly higher with 48 hours incubation compared to 24 hours incubation. (p=0.000) Vertical bars represent 95% confidence intervals.

Figure 70 Effect of full strength and diluted TSB media on biofilm formation at 25°C

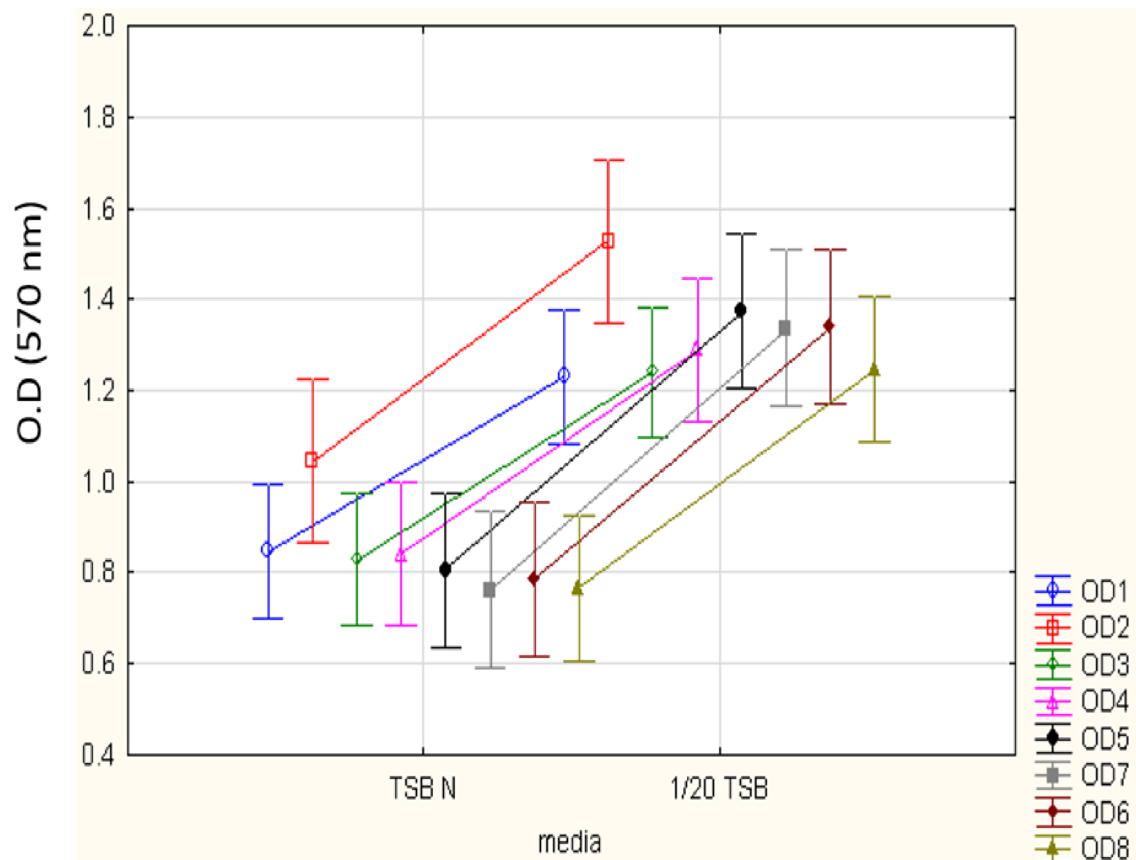


Figure 70: Effect of media on biofilm formation at 25°C: The data represents the difference in biofilm formation from 8 independent observations in full strength TSB media and 1/20 TSB medium, results indicate that biofilm production was significantly higher in 1/20 TSB media (P=0.00169). Vertical bars represent 95% confidence intervals.

5.3.3 Biofilm formation at 15°C:

The effect of time media and serotype on biofilm production at 15°C is summarised in Table 10. Results showed the same trend as demonstrated at 25°C and 37 °C, as Figure 71 revealed *S.* Senftenberg 775W produced the highest level of biofilm followed by *S.* Schwarzengrund factory and veterinary isolates. Figure 73 demonstrates that with lower temperature, biofilm formation was significantly greater after 48 hours incubation in comparison to 24 hours and results indicate media showed no difference in enhancing biofilm production. Overall no factory strains demonstrated an enhanced ability to produce biofilms in comparison to matched clinical and veterinary strains.

Table 10 Post Hoc test at 15°C to reveal the effect of time, media and serotype on biofilm formation

Effect	Wilks Value	F	Effect df	Error df	P
Time	0.253	2.421	80	801.4	0.000*
Media	0.95	0.824	8	125	0.583
Serotype	0.726	5.907	8	125	0.000*
Time*media	0.541	1.019	80	801.4	0.437
Time*serotype	0.489	1.198	80	801.4	0.123
media*serotype	0.917	1.407	8	125	0.2
Time*media*serotype	0.55	0.991	80	801.4	0.503

Table 10: a summary of the overall effect of time, media and serotype on biofilm formation at 15°C: The p values from the table indicate that incubation time (p=0.000) and serotype (p=0.000) both had a significant impact on biofilm formation.

Figure 71 Summary output produced using Factorial ANOVA highlighting differences in biofilm formation across strains at 15°C

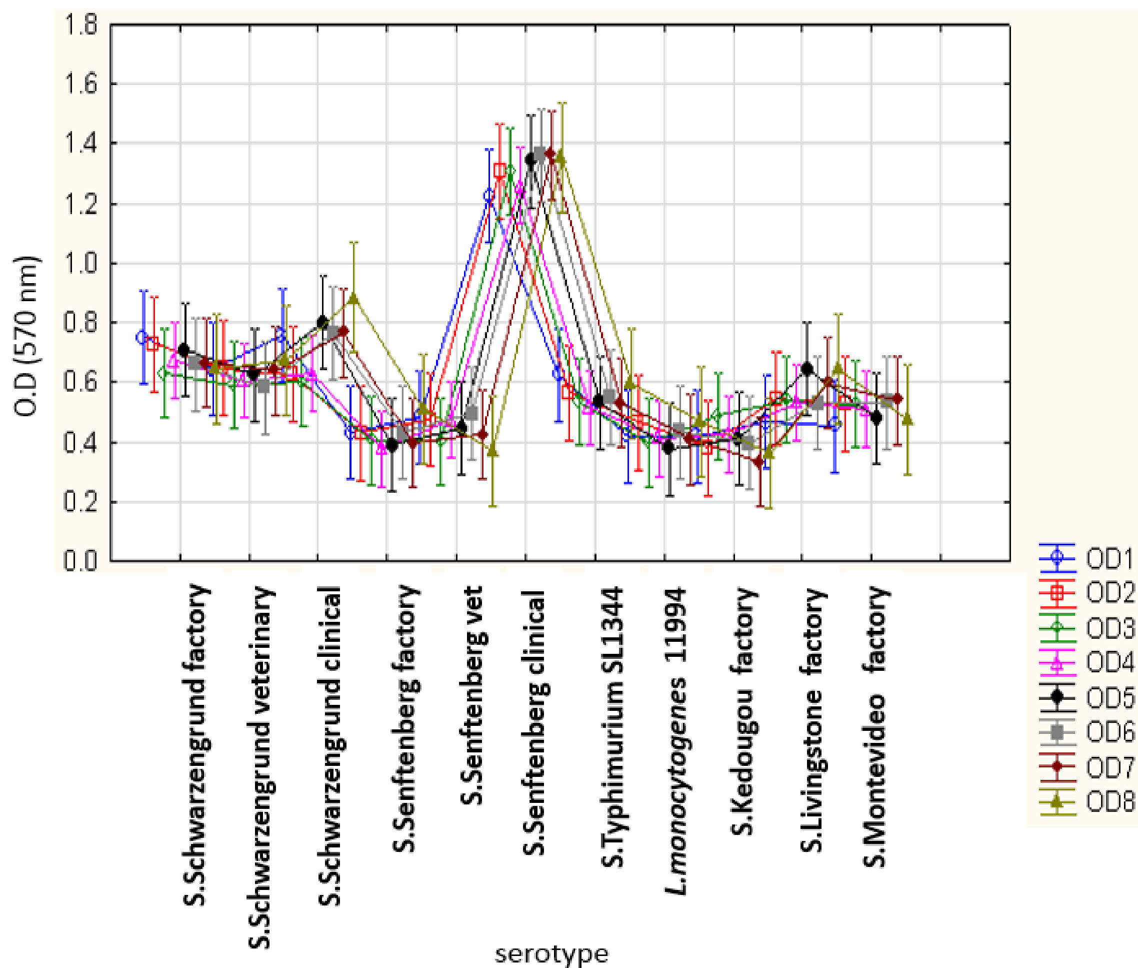


Figure 71: The factorial ANOVA output displays the mean biofilm formation for each strain and shows the overall effect of serotype and time at 15°C. Results indicated that all the strains in the panel could form biofilms and *S.* Senftenberg 775W was the strongest biofilm producer (p=0.000), followed by the factory isolate of *S.*Schwarzengrund and the veterinary isolate of *S.* Schwarzengrund. The vertical bars represent 95% confidence intervals.

Figure 72 Effect of full strength and diluted TSB medium on biofilm formation at 15°C

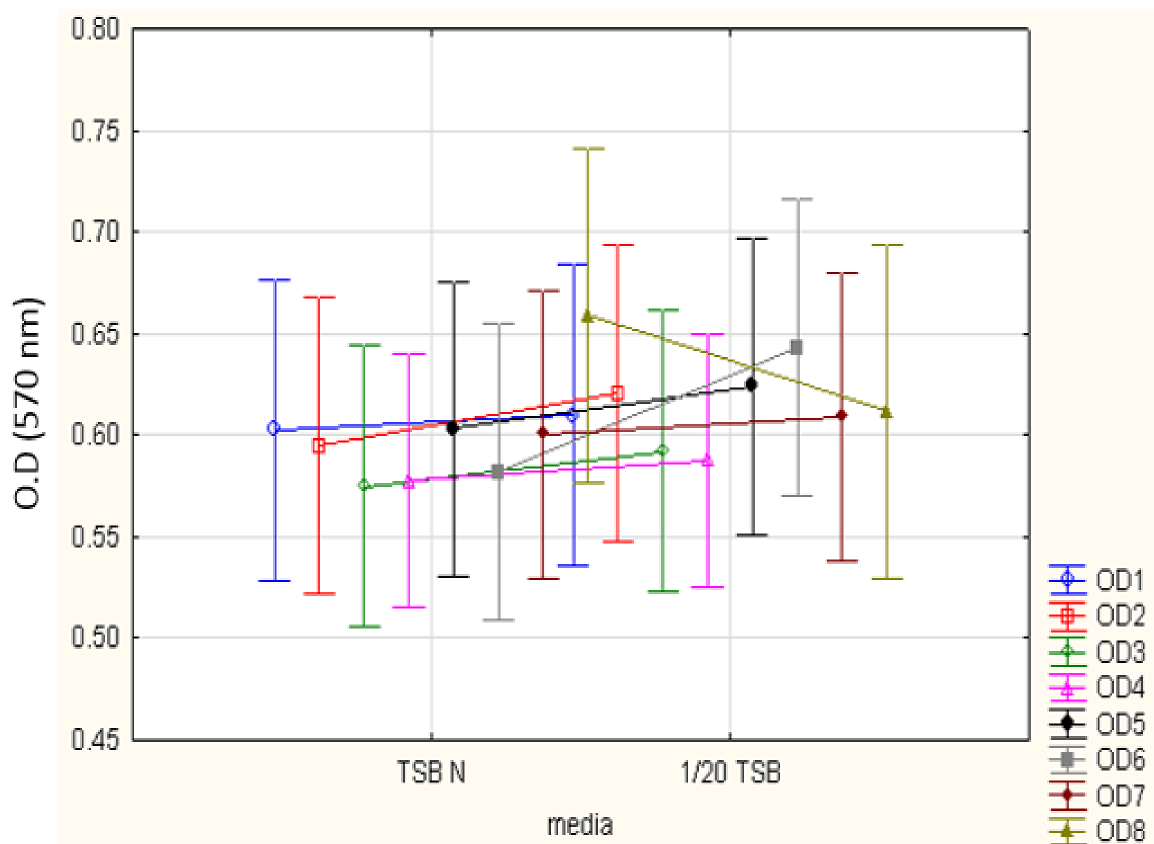


Figure 72: Effect of media on biofilm formation at 15°C. Results show that media had no significant effect on biofilm formation (p=0.57530) Vertical bars all overlapped and represent 95% confidence intervals.

Figure 73 The effect of time on biofilm production at 15°C

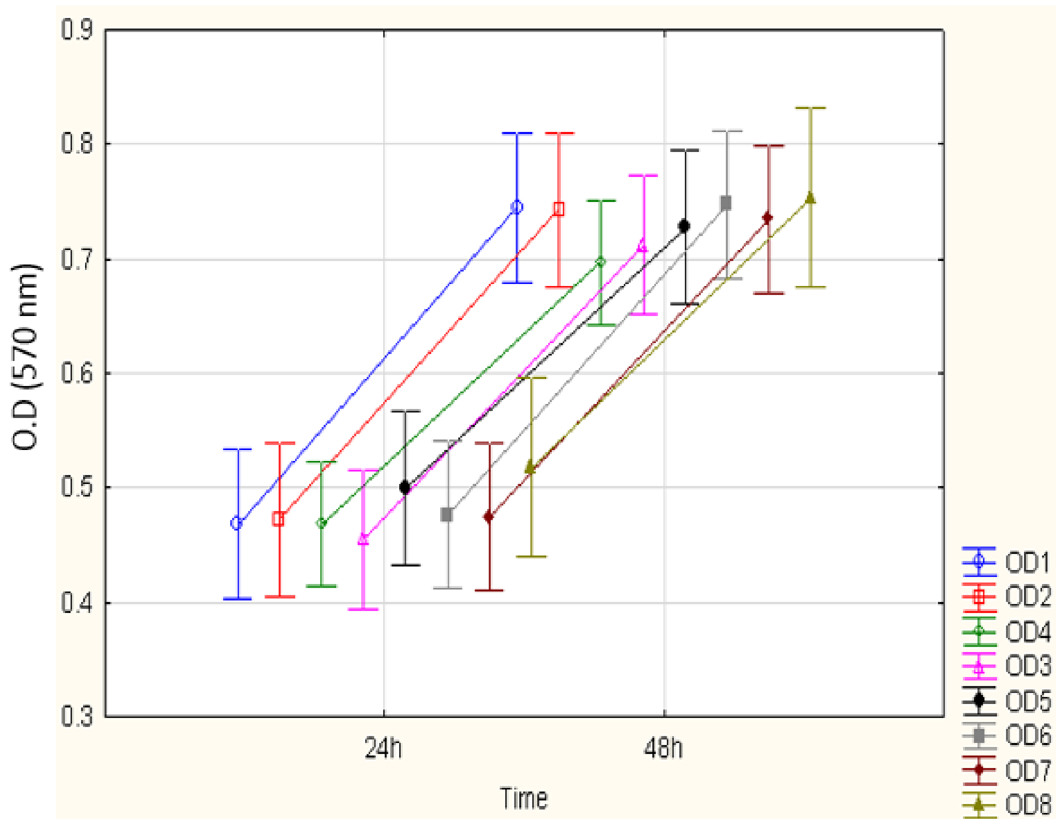


Figure 74: Effect of time on biofilm production at 15°C. A significant difference was noted between biofilm production after 24hours and 48 hours. Strains formed better biofilms with 48hours incubation (p=0.000). Vertical bars here indicate 95% confidence intervals.

5.3.4 Biofilm formation at 10°C:

In line with the other temperatures tested at 10°C, results revealed *S.* Senftenberg 775W produced the highest level of biofilm. Table 12 indicated increased incubation seemed to effect biofilm formation however the use of 1/20 TSB medium showing to have no effect on biofilm production at this temperature.

Table 11 Post Hoc test at 10°C to reveal the effect of time, media and serotype on biofilm formation

Effect	Wilks Value	F	Effect df	Error df	P
Time	0.877	2.201	8	125	0.032*
Media	0.89	1.921	8	125	0.062
Serotype	0.443	1.371	80	801.4	0.021*
Time*media	0.899	1.76	8	125	0.091
Time*serotype	0.564	0.947	80	801.4	0.61
media*serotype	0.568	0.933	80	801.4	0.643
Time*media*serotype	0.535	1.04	80	801.4	0.389

Table 11: a summary of the overall effect of time, media and serotype on biofilm formation at 10°C: The p values from the table indicate that incubation time (p=0.032) and serotype (p=0.021) showed a statistically significant effect on biofilm production. However media strength (p=0.062) did not affect biofilm production.

Figure 74 Summary output produced using Factorial ANOVA highlighting differences in biofilm formation across strains at 10°C

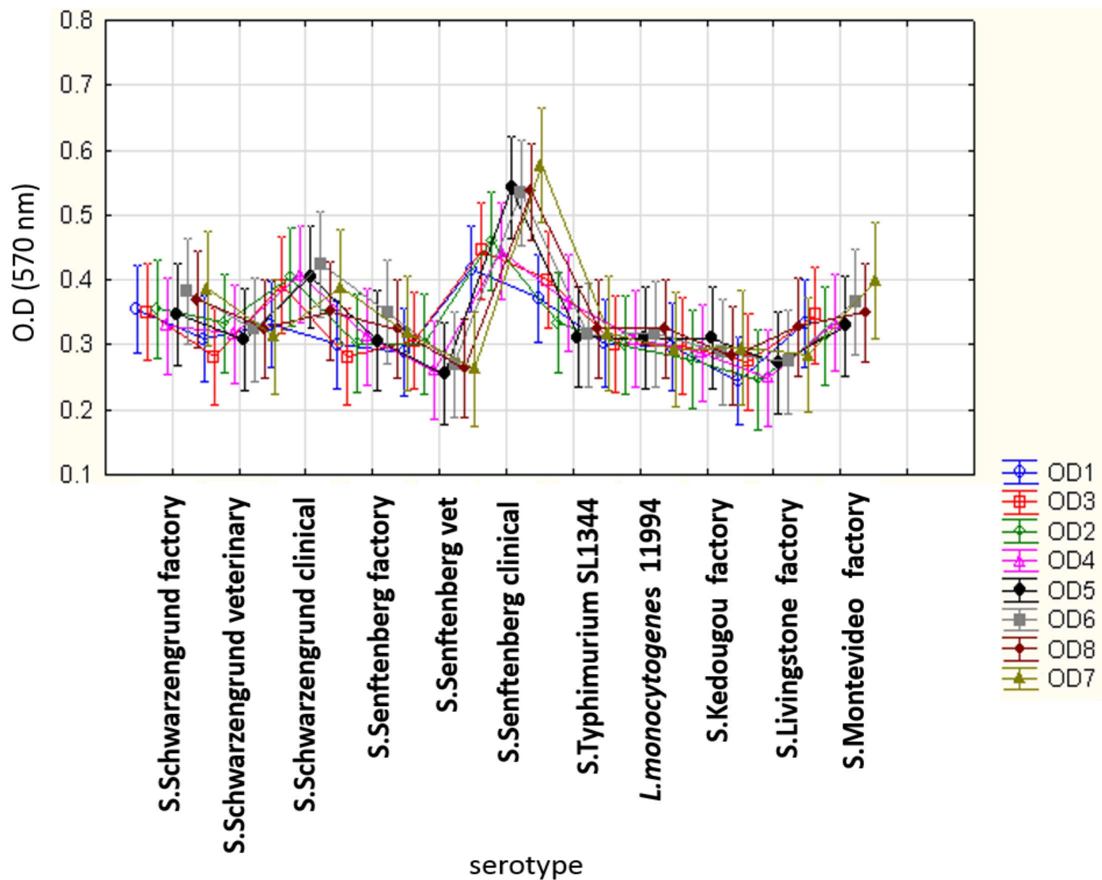


Figure 74: The factorial ANOVA output displays the mean biofilm formation for each strain and shows the overall effect of serotype and time at 10°C. Results indicated that all the strains in the panel could form biofilms and *S.* Senftenberg 775W was the strongest biofilm producer (p=0.0212), followed by the factory isolate of *S.*Schwarzengrund and the veterinary isolate of *S*. Schwarzengrund. The Vertical bars represent 95% confidence intervals.

Figure 75 Effect of full strength and diluted TSB media on biofilm formation at 10°C

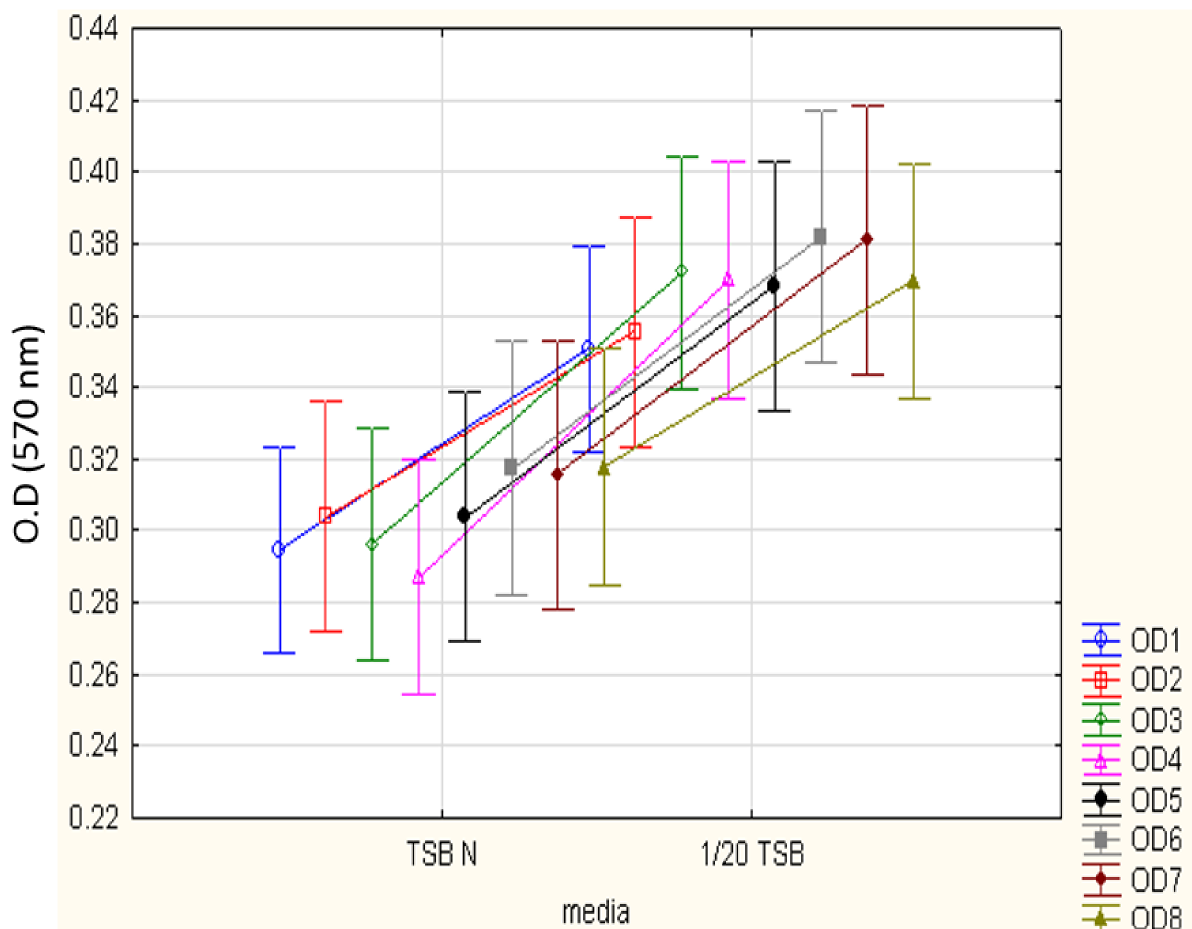


Figure 75: Effect of media on biofilm production at 10°C. Results show that in the diluted 1/20 TSB minimal medium the biofilm production was slightly higher compared to the full strength medium however this increase in biofilm was not significant (0.08466). Vertical bars represent 95% confidence intervals.

Figure 76 The effect of time on biofilm production at 10°C

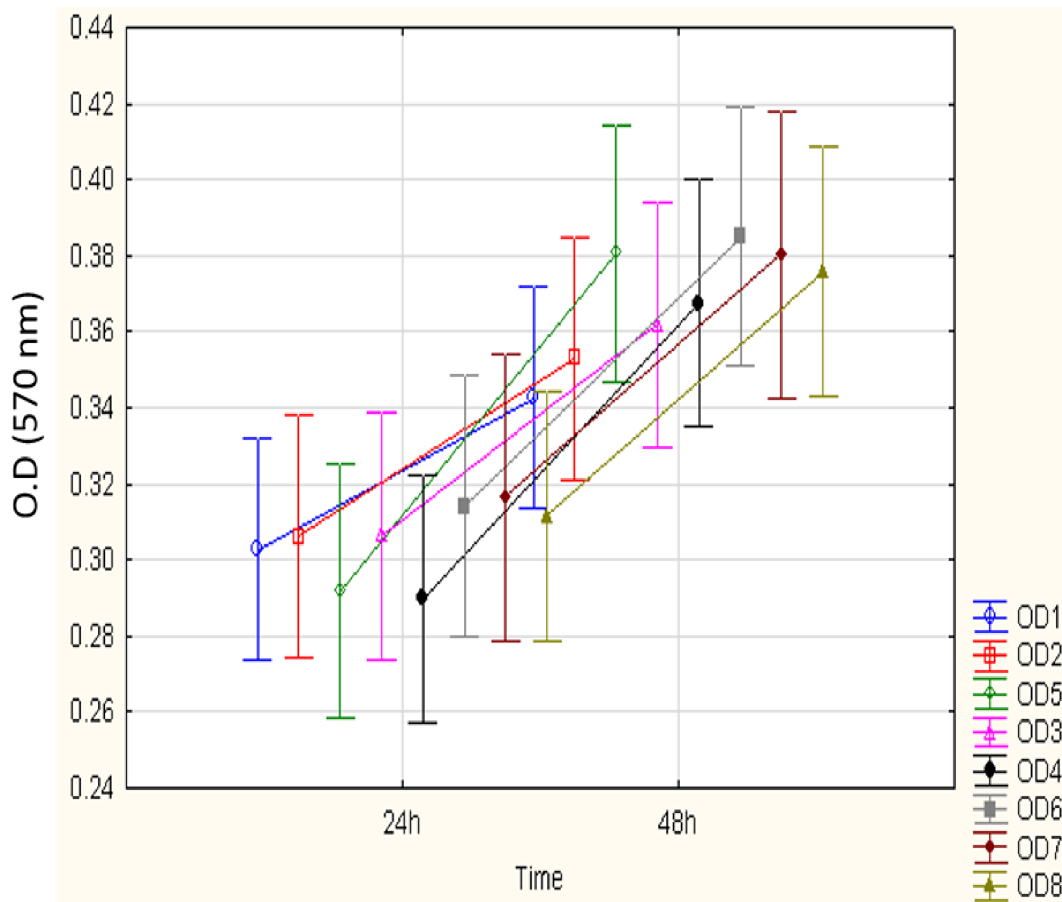


Figure 76: Effect of increased incubation time on biofilm production. Although biofilm production was higher after 48 hours compared to 24 hours, the results only show repeat 4 and 5 showed statistical significance (p=0.03009). On the other six repeats the vertical bars indicating 95% confidence intervals overlapped, revealing increased incubation did not significantly increase biofilm density.

Figure 77 Summary of the effect of environment on biofilm formation across serotype matches strains

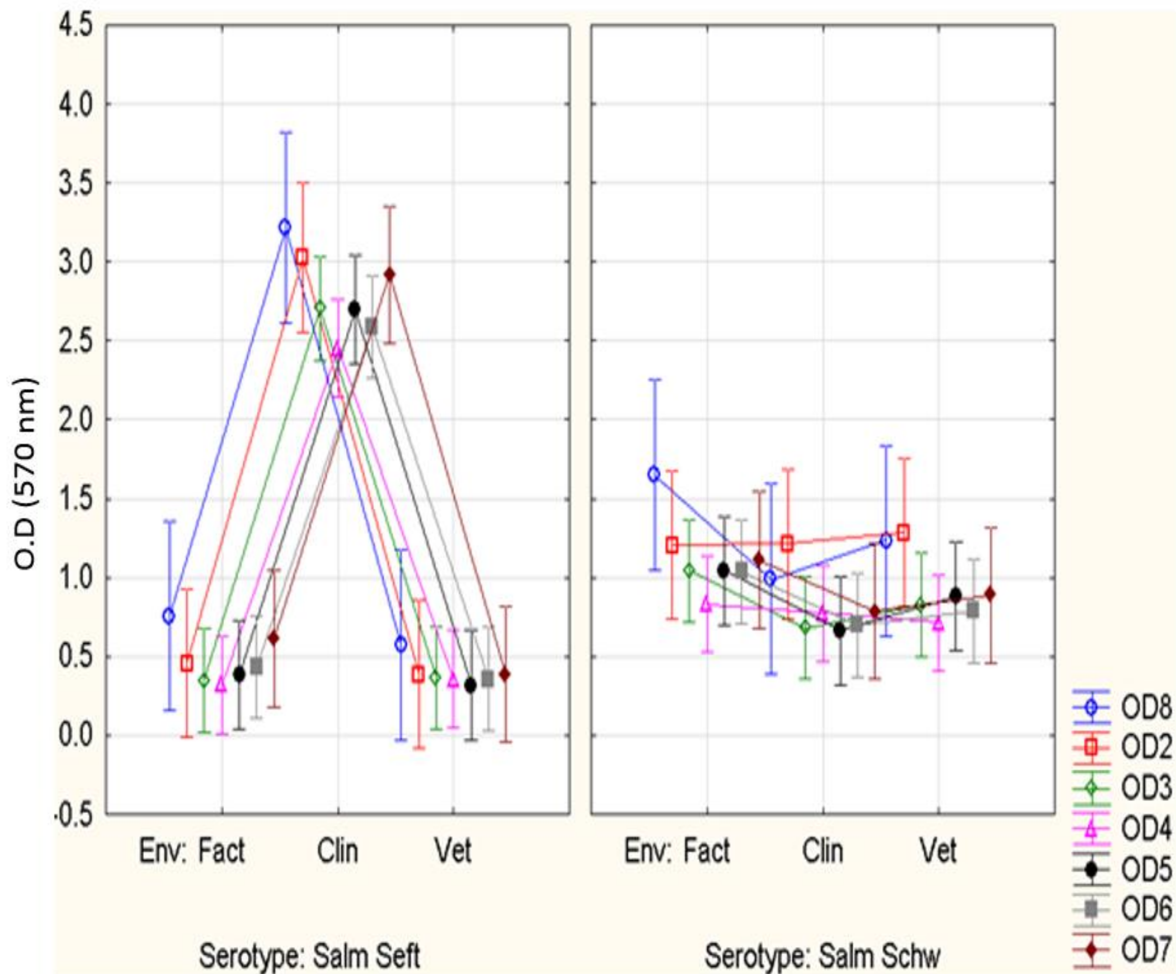


Figure 77: shows the overall effect of environment on biofilm production across all temperatures and media tested. The factory isolates of *S*.Schwarzengrund and *S*.Senftenberg were serotype-matched with clinical and veterinary isolates. The results indicate that although all strains could form biofilms, only *S*. Senftenberg 775W was statistically significantly better at forming a biofilm in all conditions tested (P=0.000). Neither of the factory strains in these groups showed any significantly enhanced ability to form stronger biofilms.

5.4 Discussion

In the food industry, biofilms pose several grave risks including manufacturing problems such as obstructing the flow of heat across a surface in addition to corrosion of surfaces which leads to both energy and production losses. Most importantly, pathogenic microorganisms like *Salmonella,* grown on surfaces have the capacity to cross-contaminate and cause post-processing contamination (Garrett *et al.*, 2008). Previous studies have highlighted that contamination of factory surfaces with *Salmonella* was a growing issue (Oliveira *et al.*, 2007; Dawson *et al.*, 2007; Margas *et al.*, 2013). Yet there is limited knowledge on the survival of resident factory *Salmonella* strains in comparison to isolates from other environments. This study attempted to analyse a panel of 11 defined isolates consisting of factory, veterinary and clinical strains and comparing their ability to form biofilms on polystyrene micro titre plates. The impact of temperature, time and media concentration on biofilm formation was also investigated.

Growing biofilms in micro titer plates and staining with crystal violet is a standard format that allows the observation of biofilm attached to the wall or bottom of the micro-titre plate. The extent of biofilm formation is determined colourimetrically by measuring the density as this correlates to the adsorption of the crystal violet in the de-staining solution. This method is relatively low cost and produces reproducible results (Djordjevic *et al.*, 2002; O'Toole, 2011).

The survival of resident factory strains in comparison to clinical and veterinary strains has not been widely investigated in previous literature; results from this study indicated that overall \`resident' factory strains did not grow significantly better biofilms than clinical and veterinary strains of the same serotype ($p=0.000$). This study also confirms all the strains investigated in the panel despite being from factory, clinical or veterinary environments could form biofilms and that biofilm formation was not serotype dependant as varying results within a serotype were observed. In contrast to findings of Stepanovic *et al.* (2004), the results indicated that there was no difference in the biofilm production of *L.monocytogenes* in comparison to a majority of the *Salmonella* strains in the panel. This is important as *Listeria* was included as a control in the study as it is recognised as a persistent pathogen that has enhanced biofilm forming abilities, but biofilm formation does vary with strain (Djordjevic *et al.*, 2002; Di Bonaventura *et al.*, 2008; Ferreira *et al.*, 2011).

Salmonella is able to persist in the food manufacturing environments for years (Nesse *et al.*, 2003). Our results suggested that *S*. Typhimurium SL1344 was not able to form biofilms as good as *S*. Senftenberg 775W and the factory isolate of *S.* Schwarzengrund. In support of these findings the weak formation of biofilms by *S.*Typhimurium has been described by others. Vestby *et al.* (2009b) reported that although *S*. Typhimurium was endemic in Norwegian wildlife and is persistent in the environment surrounding most factories, it was rarely isolated from factories and produced little biofilm in microtiter plates (Vestby *et al.*, 2009b). It also highlighted that more rare serotypes like *S.*Montevideo and *S.*Agona were good biofilm formers and *S.*Senftenberg was a medium biofilm former, moreover, these serotypes have been reported as persistent for years.

In the current study irrespective of media and temperature, only *S.*Senftenberg 775W produced biofilms at levels almost twice as high as other isolates in the panel. *S.*Senftenberg 775W is a known heat resistant strain and is used as a model organism in the food industry (Goepfert and Biggie, 1968; Ng *et al.*, 1969). This could be attributed to its ability to form biofilms as cells in biofilm demonstrate increased resistance to physical stress such as heat. Other studies comparing planktonic cells and those attached to surfaces found that cells attached to surfaces seem to be more resistant and have a raised tolerance to stress and dried cells of *Salmonella* have increased tolerance to heat (Moretro *et al.*, 2009b). A survey conducted in Norway showed that *S.*Senftenberg was the most common serovar isolated from imported feed raw materials, which could contribute to its prevalence in the factory environment worldwide as many ingredients are imported from across continents (Vestby *et al.*, 2009b), unfortunately there was little data available on the status of raw materials globally.

To our knowledge in the current literature there are no other studies that have studied serotype matched isolates from clinical and veterinary environments, although Vestby *et al.* (2009b) compared biofilm production by `persistent' and presumed non-persistent strains of *S.*Agona and *S.*Montevideo and showed that the persistent strains were better biofilm producers than the presumed non-persistent strains, the matched isolates were also from the factory environment.

Therefore, although the biofilm forming ability may be an important factor involved in the persistence of *Salmonella* globally, the factory isolates in the current study did not show a competitive advantage in biofilm density in comparison to serotype matched strains as indicated by measuring crystal violet density. Thus, opportunities for biofilm formation must be a contributing factor, such as failure in complying to HACCP control routine which enables *Salmonella* to establish and subsequently form biofilms (Vestby *et al.*, 2009b).

The temperatures selected for this particular study were obtained via environmental sampling which monitored temperature and RH from a factory site as described in chapter 4. During the factory shut down period temperatures fell to 10°C and the different zones of the factory showed temperatures ranging from 15°C to 25°C. Recently, a study was conducted by Habimana *et al.* (2010a) comparing the survival of resident flora strains in feed processing plants in response to stress factors typically found in the factory, the results reported correlated to those found in the survival on stainless steel data in the previous chapter, whereby *Salmonella* survived the least at 37°C, the longest at 25°C and cell counts rapidly declined at 10°C.

Interestingly, biofilm formation was also highest at 25°C, followed by 37°C and decreased respectively at 15°C and the lowest level of biofilm formation was seen at 10°C. This indicated that at higher temperatures, close to the human body temperature *Salmonella* survived better in a biofilm, generally enteric bacteria grow better at 37°C and if cells can establish and grow, then biofilm formation rate is likely to be better (Stepanovic *et al.*, 2004). At lower temperatures the strains were unable to form as strong biofilms, presumably as cells struggled to grow; if cells were unable to grow they could not attach to a surface and grow in number to produce extracellular matrix. Similarly, a study by Tammakritsada & Todhanakasem (2012), investigated the ability of *Salmonella* to form biofilms on polystyrene tubes and also showed that the same pattern with biofilm levels decreasing with temperature from 25°C to 15°C and finally to 10°C. At 25°C, results indicated that *Salmonella* could make better biofilms after 48 hours which is also supported by the study conducted by Stepanovic *et al.* (2004). This poses as a potential risk factor for food factories as *Salmonella* is able to form biofilms on surfaces and survive for months at 25°C which is close to factory ambient temperature and these biofilms pose a major risk of cross- contamination of food products.

Bacteria persisting in the food processing environments are likely to be exposed to differing levels of available nutrients depending on their location in a factory plant (Djordjevic *et al.*, 2002). In addition in laboratory studies it is well established that the concentration of the culture medium is an important variable in influencing the growth and biofilm formation for

both *Salmonella* and *L.monocytogenes* (Stepanovic *et al.*, 2004). Furthermore time is also an important parameter in biofilm development; the longer bacterial cells have to form biofilms, normally the more comprehensive and dense the biofilm is. Stepanovic *et al.* (2004), investigated biofilm formation in four media types at 35°C over 24 hours; brain heart infusion (BHI), tryptic soya broth (TSB), meat broth (MB) and 1/20 diluted trypcase soya broth. The results highlighted that *Salmonella* formed better biofilms in low nutrient diluted TSB media (which was used to mimic factory conditions) in comparison to full strength TSB.

In the current study, biofilm formation in 1/20 TSB medium was compared to that in full strength TSB medium at four temperatures and the effect of increased incubation to 48 hours following a media change was also investigated. In line with these data, all the isolates were able to form biofilms in TSB at full and 1/20 concentration at 25°C and 37°C. The lower nutrient content influenced biofilm production, producing significantly higher levels of biofilm production.

Joseph *et al.* (2001) investigated the ability of poultry isolates of *Salmonella* to form biofilms on stainless steel, plastic and cement, and found that the highest density of biofilm formed on plastic, followed by cement and stainless steel. Other studies also indicated that *Salmonella* and *L.monocytogenes* adhere in higher numbers to hydrophobic material such as plastic (Sinde and Carballo, 2000; Donlan and Costerton, 2002). Considering adhesion is the primary step in biofilm formation, it could explain why all the isolates were able to form good biofilm on plastic surfaces (Stepanovic *et al.*, 2004).

In the current study, the biofilm forming ability of pure cultures of *Salmonella* isolated from factory, veterinary and clinical environments was investigated and they were all found to produce biofilm; it was important to bench mark the biofilm producing capabilities of the panel of isolates as pure cultures prior to more in depth investigation. It is recognised that the factory micro-biome is likely to contain competing organisms that are coexisting with *Salmonella* and other research has suggested that a synergistic effect may have an influence on biofilm persistence. Habimana *et al.* (2010b) showed that biofilms of *S*. Agona were supported in a mixed species biofilm with both *Pseudomonas* species (3.2 fold increase) and *Staphylococcus* spp. (2.8 fold increase) (Habimana *et al.*, 2010b).

5.5 Conclusion

Biofilm formation may serve as a potential reservoir for the persistence of *Salmonella* in the environment. Results from the current study highlighted that all the isolates in the challenge panel were able to form biofilms in both nutritious and nutrient limited environments and temperature played an important role, with higher levels of biofilm production occurring at 25°C and 37°C. At 37°C an extended duration of incubation had no beneficial effect on the ability of strains to form more established biofilms however at 25°C, 15°C and 10°C more established biofilm formation was correlated with increased incubation of 48 hours. None of the factory isolates showed an enhanced capability to form biofilms in comparison to serotype-matched isolates from veterinary and clinical sources. Therefore it is unlikely that biofilm formation in isolation is responsible for the environmental persistence observed in the food isolates, however it is likely to play a contributory factor in other persistence strategies such as resistance to biocides, desiccation or other environmental extremes.

6 Chapter 6 Investigating the efficacy of chemical agents against the panel of isolates

6.1 Introduction

The persistence and control of *Salmonella* spp.in low moisture foods is an important challenge for the food industry (Finn *et al.*, 2013a). It is well established that *Salmonella* are readily able to form biofilms on a range of surfaces present in the food manufacturing environment. Previous studies have modelled conditions of the food factory environment such as temperature and nutrients whist investigating biofilm formation on a range of surfaces including; plastic, steel, concrete, rubber and tiles (Vestby *et al.*, 2009b; Corcoran *et al.*, 2014). As discussed in chapter 5, all the isolates in the panel are capable of biofilm formation, with stronger a stronger density of biofilm formation in nutrient limited media with an extended incubation of 48 hours. This is concerning as in favourable conditions the surviving cells may regrow and result in the cross- contamination of equipment and food products. Importantly, the food factory isolates tested in the panel were not found to produce enhanced biofilms in comparison to isolates from clinical and veterinary environments, suggesting that environment is not a major factor contributing to enhanced biofilm formation. Previous work has implied that the persistence of *Salmonella* is associated with biocide resistance as cells in biofilm are able to withstand penetration of antimicrobial agents unlike planktonic cells (Spoering and Lewis, 2001; Braoudaki and Hilton, 2004; Smith and Hunter, 2008).

Cells in biofilm are more difficult to eradicate as they have a diversity of defence mechanisms and unlike in their planktonic state the cells are 10 to 1000 times more resistant to antimicrobials (Costerton *et al.*, 1995a; Moretro *et al.*, 2009a). Despite biofilm removal occurring naturally via intrinsic processes, in the food industry mechanical removal through the use of disinfectants and biocides is a common process. The mechanism by which microorganisms develop resistance is not fully understood however it has been suggested that the disinfectant may not be able to penetrate the EPS or may chemically react with outer layer of the biofilm and this interaction quenches the antimicrobial activity before reaching the cells embedded in the matrix. This leads to reduced diffusion and interaction with viable cells and perhaps inactivation of the disinfectant. Moreover, fluctuations in normal environmental conditions cause bacteria to employ survival strategies, resulting in cellular changes such as a reduced growth.

Microorganisms that are starved for nutrients shift from exponential growth to either slow or no growth, and this change is usually accompanied by an increase in antibiotic resistance (Araújo *et al.*, 2013). Additionally, it has been reported that exposure to different environments resulted in differing growth rates in cells within the same biofilm and bacterial resistance is dependent on their state, with cells in the stationary phase exhibiting a higher resistance (Mah and O'Toole, 2001).

Moreover, changes in the permeability of the cytoplasmic membrane and the composition of the cell wall serve to prevent disinfectant entry. Exposure to sub- lethal levels of biocides results in minor cellular damage as changes in metabolic activity are evoked as well as an up regulation of genes responsible for biofilm formation, giving rise to a more resistant population (Araújo *et al.*, 2013; Corcoran *et al.*, 2014).

According to The European Standard released on 24th April 1998 (CE/8/98), ``biocidal products are active substances, or preparations that contain one or more active substances, that are presented to the user in their final form, and whose function is to either destroy, stop the growth, make harmless, avoid or control by any mean the action of a pathogenic organism by a biological or chemical process''(Araújo *et al.*, 2013). Microbial control programmes such as GMP and HACCP introduce plans that eradicate, or decrease microorganisms and their activity to a satisfactory level in addition preventing and controlling the formation of biological deposits on processing equipment. However, adherence to cleaning and disinfection protocols using the correct agent combined with the required contact time, in the correct frequency is essential in preventing biofilm build up in manufacturing facilities.

Currently an array of chemical compounds are available for disinfection some of which include; alcohols, aldehydes, biguanides, phenols, acids, and quaternary ammonium compounds (QACs) (Araújo *et al.*, 2013). Each antimicrobial has a different mechanism of action depending on its cellular target. Despite many antimicrobial agents being marked effective against *Salmonella,* these tests are based on suspension tests which are only relevant in studying the mechanisms by which disinfectants enter and kill cells. To model real events that may occur in a factory niche, it is essential to understand more about the efficacy of antimicrobials when applied to biofilms that are attached to a surface as well as the effect of contact time (Moretro *et al.*, 2009b; Corcoran *et al.*, 2014).

6.1.1 Quaternary ammonium compounds/ Benzalkonium chloride

Quaternary ammonium compounds (QACs) belong to the cationic detergent group and are commonly used disinfectants in the food and medical industry. At lower concentrations they target membrane permeability by penetrating the cell wall, reacting with the membrane which causes membrane disorganisation, leading to cytolytic leakage of cytoplasmic material, degradation of proteins and loss of structural integrity, whereas at higher concentrations, they target the carboxylic groups, causing general coagulation in the bacterial cytoplasm. The QACs are effective against a range of organisms including Gram-positive bacteria, Gram-negative bacteria, some viruses, protozoans and fungi (To *et al.*, 2002; Braoudaki, 2004). Benzalkonium chloride (BKC) is a widely used quaternary ammonium compound that distorts the cytoplasmic membrane by reacting with phospholipids. It is a product of the nucleophilic substitution reaction of alkyldimethylamine with benzyl chloride (Fazlara and Ekhtelat, 2012).

6.1.2 Chlorhexidine

Chlorhexidine, a cationic bis-biguanide, has been used as an antimicrobial agent since 1953. It exhibits a wide spectrum of bactericidal activity but due to its cationic characteristic its activity is reduced with soaps and other anionic compounds (McDonnell and Russell, 1999). It has a similar mode of action to BKC and is capable of killing bacteria by distorting the cytoplasmic membrane by reacting with the phospholipid layer, causing membrane damage followed via intracellular coagulation. Once the cell membrane is damaged it causes leakage of cell components however the damage does not result in cell lysis. It is often used in hospitals as a surgical scrub and skin disinfectant and it has been used as a mouthwash to treat periodontal disease in clinical settings. Ten to fifty times the MIC concentration is used in order to kill 99.9% of bacteria within 10 minutes at 20°C. Chlorhexidine is more effective against Gram positive bacteria in comparison to Gram negative, mainly due to the permeable cell wall of Gram positive organisms (Grossman *et al.*, 1986; Braoudaki, 2004; Russell, 2012). Shen *et al.* (2011) investigated the effect of chlorhexidine on 2 day old and 3 week and 12 week old biofilms and showed that chlorhexidine was less effective in killing bacteria in mature biofilms and those grown in nutrient-limited biofilms in comparison to young biofilms (Shen *et al.*, 2011).

6.1.3 Sodium hypochlorite/calcium hypochlorite

The hypochlorites are the most frequently used chlorine products (or chlorine releasing agents CRA), they are typically aqueous solutions of 5.25%–6.15% and often usually referred to as household bleach. The hypochlorites exhibit a broad spectrum of non-specific killing against an array of microbes, the preparations are also sporicidal and viricidal. They are commonly used to disinfect hard surfaces (McDonnell and Russell, 1999), they tend not to produce toxic residues and are rapid. However their effectiveness against cells in biofilms attached to surfaces is debatable, with some studies showing a high cell reduction (Cabeça *et al.*, 2012) with others showing a minimal effect (Gandhi and Matthews, 2003). Although as a group chlorine releasing agents have been widely studied, the exact mechanism of action is still unclear; they are highly active oxidizing agents, the antimicrobial properties of NaOCl are based on in its high pH (hydroxyl ion action). It acts by interfering with the integrity of the cytoplasmic membrane, denaturing the cellular activity of proteins, causing biosynthetic alterations in cellular metabolism as well as phospholipid degradation (McDonnell and Russell, 1999; Hamed *et al.*, 2014). Vestby *et al.* (2009b) tested the efficacy of 0.05% sodium hypochlorite against *Salmonella* biofilms incubated for 48 hours; results showed a mean reduction of 2.4 log_{10} CFU/ml of *S. enterica* cells recovered from the surface following five minutes of exposure (Vestby *et al.*, 2009b).

6.1.4 Peracetic acid

Peracetic acid (PAA) ($C_2H_4O_3$) is produced from a chemical reaction between acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2). It is a multipurpose disinfectant used to control microbial contamination in food, clinical settings and water. In the food industry it is used to remove both bacteria and fungi from food products, PAA sparked research interest because it breaks down to acetic acid, oxygen and water making it practically non- toxic to food and the environment (Block, 2001). It is also used to disinfect medical equipment in hospitals and in the water industry for cooling tower disinfection; it is used to prevent biofilm formation and *Legionella* contamination. Peracetic acid functions like other as other oxidizing agents by primarily targeting the cell membrane, denaturing proteins, disrupting the permeability of the cell wall (Kitis, 2004; Bauermeister *et al.*, 2008; Russell, 2012). However, PAA treatment is expensive due to its limited production capacity worldwide.

6.1.5 Tego 2000

Tego 2000 is a mixture of an amphoteric surfactant and a cationic detergent meaning they are less affected by pH changes compared to other disinfectants and they are often compared to the QAC's. It serves as a general disinfectant and sanitizer against both Gram positive and Gram negative bacteria and has extensively been used in the food and beverage industry in the past forty years as well as the clinical setting for sterilization of medical equipment (Block, 2001). In the literature the antimicrobial activity of Tego 2000 has not been widely studied, however research with other Tego based products have revealed resistant Gram negative rods in an animal laboratory, indicating that bacteria may adapt to and survive Tego disinfection when conditions are not optimal (Kellett, 1979). Furthermore Langsrud *et al.* (2003) found that *Serratia marcescens* was isolated from disinfecting footbaths containing TEGO 103G (amphoteric disinfectant) in five of six dairy factories. The isolates from disinfecting footbaths did not achieve a ≤ 5 $\log_{10}$ reduction with the recommended concentration of TEGO 103G, TEGO 51 or benzalkonium chloride. However all strains were effectively killed with disinfectants based on peracetic acid, hypochlorite, quaternary ammonium compounds and alkyl amino acetate (Langsrud *et al.*, 2003).

6.1.6 Sorgene

Sorgene is a combination of peracetic acid and hydrogen peroxide and is used as a broad spectrum disinfectant in farm conditions against bacteria, viruses & fungal spores. It is also the approved disinfectant against foot and mouth disease and poultry (BASF, 2013). Oxidative biocides like chlorine and hydrogen peroxide (H_2O_2) act by removing electrons from susceptible chemical groups, oxidizing them, and becoming reduced in the process. This causes damage to the surface, cell wall as well as intracellular damage. Being low molecular weight compounds allows easy passage through cell walls/membranes, leading to internal damage when the agents react with internal cellular components, which may result in optotic and necrotic cell death. Although the specific mode of action varies amongst oxidising agents the physiological actions are similar and they have numerous targets within a cell as well as in almost every biomolecule (Finnegan *et al.*, 2010).

6.1.7 Virkon

Virkon is a blend of peroxygen compounds, surfactant, organic acids and an inorganic buffer (Dupont, 2009). Its mode of action is based on oxidation of proteins and constituents of cell protoplasm, which lead to the inhibition of enzyme systems and loss of cell wall integrity. Previously, Moretro *et al.* (2009b) investigated the efficacy of that Virkon against 2 day old biofilms of *S.* Agona and *S.* Senftenberg grown on stainless steel surfaces following 2 days of incubation at 20°C, results revealed complete reduction (>4 log) in cell count (Moretro *et al.*, 2009b).

One of the challenges faced in the food industry is that many plants require dry conditions, meaning cleaning with water and disinfectants may not be routinely performed, which could lead to a build-up of organic matter (dust and residues), and studies have shown disinfectant activity is reduced in the presence of organic matter (Moretro *et al.*, 2009b). Although other studies have investigated efficacy of different classes of disinfectants against *Salmonella* biofilms, it was important to know if the disinfectants currently being used in the food factory were effective against factory isolates in the current panel and whether the factory strains were less susceptible to the products in comparison to serotype matched isolates. Furthermore adherence to protocol contact times is key for efficient removal of cells in biofilm; different plants have variations in contact time length, so it was important to establish the effect of a shorter exposure time, as in reality when cleaning equipment/ wiping surfaces, the disinfectant may be wiped off earlier than the recommended times.

Therefore, the aim of this chapter was to determine the susceptibility of the panel of isolates to a range of disinfectants typically used in the food industry in addition to determining the ability of these disinfectants to penetrate through two day mature biofilms grown in microtiter plates.

6.2 *Methods and materials*

6.2.1 *Minimum Inhibitory Concentration (MIC) in 96 well plate format*

Minimum Inhibitory Concentrations (MIC) was determined producing a profile for each strain by performing the standard micro broth dilution method (WHO, 2010).

6.2.2 *Preparation of bacterial suspension*

The MIC of each chemical agent was performed in a 96 well micro titer plate format and two isolates were tested in each plate. *Salmonella* isolates were resurrected from -80°C storage and a single bead was used to inoculate 10mL Nutrient Broth (Oxoid, Basingstoke, UK) which was incubated for 24 hours at 37°C in an orbital shaker (Orbital shaking incubator, Gallenkamp) set at 200rpm. The inoculation was standardised by counting cells in a haemocytometer and the concentration adjusted using sterile nutrient medium to give a final concentration of 10^5 cells/ml.

6.2.3 *Preparation of biocide concentrations*

The biocide stock solutions were prepared in sterile 20ml plastic universals; a concentration of 1% w/v was made for each of the biocides. This was achieved by adding 0.2g of biocide agent into 19.8ml of SDW. This was then designated the `neat' stock solution. In the 96 well plate, biocide concentrations were made up by doubling dilutions, 300 µl of the neat biocide was added to the first well of the 96 well plate, in the remainder of the wells, 150 µl of SDW was added. A 150 µl of neat biocide was then taken from the first well and added to the well containing 150 µl of SDW and mixed by irrigation using a pipette, this concentration was labelled 1/2. This was repeated, creating the doubling dilution until a final concentration of 1/32000 was achieved.

6.2.4 *MIC in 96 well plate format*

Following the preparation of biocide concentration, media and bacterial suspension were added to each well. Each test well was made up of 144µL of double strength TSB, 6µL of the adjusted bacterial suspension and 150µL of the biocide concentration being tested. Biocide free inoculum was used as a control. A separate control plate was created repeating the above procedure without inoculum to ascertain effect of biocides on the turbidity of TSB. The plates were incubated at 37°C for 24 hours and the absorbance was read at 570nm using the Biotek

ELx808 Absorbance Microplate reader. All experiments were conducted in quadruplicate and three readings were taken for each plate. Absorbance readings were calculated by removing the control values for each dilution The MIC was determined as the lowest concentration of the antimicrobial agent required to inhibit the growth.

6.2.5 Biofilm disinfection assay

The susceptibility of cells in biofilm was investigated using a modified version of the European surface test, used by (Moretro *et al.*, 2009b). Six strains were selected (out of the eleven) for biofilm disinfectant testing; which included the factory isolates of *S.*Schwarzengrund, *S.*Senftenberg and *S.*Livingstone, veterinary isolate of *S.*Schwarzengrund, *S.*Schwarzengrund (American human clinical isolate FSL S5-458) and *S.*Typhimurium (SL1344). Biofilms were produced in full strength TSB media as described in chapter 5. Following 48 hour incubation at 37°C the medium was removed using a multi -channel pipettor and the well washed twice by irrigation with SDW three times. Next 250µl of the 1% disinfectant was added to the well and incubated at room temperature for either 1 minute or 5 minute contact time. Following the desired contact time the disinfectant was removed and immediately replaced with 300 µl Dey and Engley neutralising broth (D/E neutralising broth) (Fisher Scientific, UK). D/E Neutralizing Broth neutralizes the inhibitor action of disinfectant carryover, permitting differentiation between bacteriostasis and the true bactericidal action of disinfectant chemical. This was allowed to stand for 20 minutes at room temperature. Using a sterile pipette tip any remaining biofilm was scraped off the sides of the well and dislodged into the D/E neutralising broth. This was assigned as the `neat`. Serial dilutions were performed in sterile 0.85% w/v Saline solution and 100µl of the suspension was inoculated onto the surface of a Nutrient agar plate. The plates were incubated at 37°C for 24 hours and cfu/ml was recorded. All experiments were conducted in triplicate.

6.2.6 Alternative method for biofilm production

Nutrient broth, Mueller-Hinton agar (MHA) and Tryptic soy agar (TSA) were purchased from Fisher Scientific (Loughborough, U.K.), prepared according to the manufacturer's instructions and sterilising by autoclaving at 121 °C for 15 minutes. The agar was cooled to 50 °C before pouring into sterile petri dishes and allowed to set, and the broth was stored at 4 °C until required.

The six strains described in section 6.2.5 were selected for biofilm penetration testing. Overnight cultures of the six isolates were produced by adding a microbead into 10ml of fresh nutrient broth and incubated in a shaking incubator at 37°C for 24 hours to give 10^8 CFU/ml. This was then diluted in sterile saline to give approximately 10^4 CFU/ml. Tryptic Soy agar (TSA) was used to support the growth of the biofilm.

A method adapted from Al-Fattani & Douglas (2004) was used to test the penetration of biocide through a biofilm grown on a membrane. Polycarbonate membrane filters (diameter, 25 mm; pore size 0.2 µm; Whatman, UK) were sterilised by exposure to UV (15 minutes per side) and aseptically placed in the centre of the TSA plates using sterile forceps. A Fifty microliter volume of the 10^4 CFU/ml culture was pipetted onto the membrane filter and plates were incubated overnight at 37°C. Following incubation, the membrane with the biofilm was transferred onto a new plate of TSA, and again incubated for a further 24hours. Membrane supported biofilm therefore had a total incubation time of 48 hours for biofilm formation.

6.2.7 Biofilm Susceptibility

The biofilms were then transferred to a disinfectant containing agar (MHA and Tego 2000). The disinfectant suspension was prepared immediately prior to use by adding 20ml of Tego 2000 disinfectant to molten culture medium (MHA) at 50°C, equating to a concentration of 66 times the MIC. A concentration disk moistened in saline was placed on top of the biofilm. A 13 mm filter was sterilised by autoclaving at 121°C and placed on top of the biofilm to protect the disinfectant disk from the biofilm (Al-Fattani and Douglas, 2004). This was incubated 18-24 hours at 37°C.

Figure 78 A schematic representing the ability of disinfectants to penetrate a biofilm formed on a Polycarbonate membrane filters

Figure 78: schematic showing biofilm experiment to determine disinfectant penetration. The biofilm (A) was developed on a 25mm diameter membrane (D) resting on TSA. A blank 25mm diameter membrane (C) was placed on top of the biofilm, with a moistened disk (B) placed on top of the 13mm membrane. The membrane supported biofilm (components A-D) was transferred to disinfectant containing MHA (E).

6.2.8 Disinfectant Penetration

The disingfectant capture disc was removed from the biofilm and placed on nutrient agar that had been inoculated with 10^4 cfu/ml and incubated overnight at 37°C. The zone of inhibition was measured and used to determine the concentration of active disinfectant that had penetrated through the biofilm.

6.2.9 Determining initial Biofilm load

The biofilm was released from the membrane by mixing by vortexing in 10ml of saline for 2 minutes. This recovered suspension was designated the neat. Serial dilutions were subsequently performed from this neat solution from 10^{-1} to 10^{-4} suspension and 100 µl of each inoculated onto the surface of a nutrient agar, the inoculated plates were incubated for 18-24 hours at 37 degrees, following which colonies were counted and recorded.

6.3 Results

6.3.1 MIC determined by micro broth dilution

The minimum inhibitory concentrations of the chemicals tested were determined using the micro broth dilution method and recorded in Table 12. An adjacent column was created next to the MIC result which listed the manufacturer's recommended concentration and whether the MIC results were sensitive or resistant.

Table 12 The MIC values of chemical agents against planktonic cells

strains	**MIC Sodium Hypochlorite (%)**	**MRC** 5.25-6.15%	**MIC Sorgene (%)**	**MRC** 1%	**MIC Biosan (%)**	**MRC** 3.5-5%	**MIC Tego 2000 (%)**	**MRC** 0.5-2%	**MIC Peracetic Acid (%)**	**MIC Virkon (%)**	**MRC** 1%	**MIC Chlor-hexadine (%)**	**MIC BKC (%)**	**MRC** 0.01-0.05%
S.**Senftenberg Clinical**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00305	**S**	0.0976	1	**S**	0.25	0.0313	**S**
S.**Senftenberg Factory**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00305	**S**	0.09765	1	**S**	0.25	0.0625	**S**
S.**Senftenberg Vet**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00305	**S**	0.09765	1	**S**	0.125	0.0156	**S**
S.**Schwarzengrund Clinical**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00610	**S**	0.09765	1	**S**	0.125	0.0313	**S**
S.**Schwarzengrund Vet**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00610	**S**	0.04882	1	**S**	0.125	0.0156	**S**
S.**Schwarzengrund Factory**	0.39	**S**	0.001	**S**	0.003052	**S**	0.00305	**S**	0.0488	1	**S**	0.0156	0.125	**S**
S.**Kedougou factory**	0.78	**S**	0.001	**S**	0.003052	**S**	0.0030	**S**	0.0976	1	**S**	0.125	0.0313	**S**
S.**Livingstone factory**	0.39	**S**	0.001	**S**	0.003052	**S**	0.0030	**S**	0.0488	1	**S**	0.25	0.0156	**S**
S.**Montevideo factory**	0.78	**S**	0.001	**S**	0.003052	**S**	0.00305	**S**	0.0976	1	**S**	0.125	0.0156	**S**
S.Typhimurium SL1344	0.39	**S**	0.001	**S**	0.001526	**S**	0.0015	**S**	0.0488	1	**S**	0.125	0.0156	**S**
L.monoctyogenes	0.39	**S**	0.001	**S**	0.000763	**S**	0.0030	**S**	-	-	-	-	-	-

MRC= Manufacturers recommended concentration **S=Sensitive** **R=Resistant** **- not tested**

Table 13: displays the MIC values of the panel of chemicals against planktonic cells. The manufacturers recommended concentrations are stated in the column adjacent to the MIC values obtained, the `S' represents sensitive. There was little information available on the recommended concentrations of Chlorhexidine and Peracaetic acid as they are not commonly used as sole agents for disinfection. The results indicate that 0.39% Sodium hypochlorite, 0.01% Sorgene, 0.003% Biosan and 0.003% Tego 2000 were sufficient to inhibit growth after incubation with the bacterial suspension for 24 hours. Whereas some varying MIC results were observed with the other agents; a concentration of 0.04-0.09% peracaetic acid, 1% Virkon, 0.125% Chlorohexidine and 0.0156% Benzylkonium chloride were sufficient to inhibit all bacterial growth after incubation with the bacterial suspension for 24 hours at 37°C.

There was slight variation across the MIC results between isolates but no major differences were observed. The MIC for Sorgene, commonly used as a farm disinfectant was 1000 times less than the recommended 1%. Tego 2000 is used as a factory disinfectant; again the recommended concentration was 0.5-2% however the MIC was significantly lower. The MIC of Virkon was at manufacturers recommended concentration of 1%.

*Due to time constraint no MIC results are available for Virkon, Chlorohexidine, Benzylkonium chloride and Peracaetic acid against *L.monocytogenes.*

6.3.2 Assays investigating disinfection penetration through Salmonella biofilm

The susceptibility of six disinfectant agents which consisted of Tego 2000, BKS, CHX, NACLO, Virkon and PAA were tested against cells in biofilm using a modified version of the European surface test. Six *Salmonella* strains out of the panel of eleven were selected and grown in full strength TSB medium in a microtiter well plate using the method described in chapter 5. The disinfectant agent was allowed either 1 minute or 5 minute contact time after which remaining biofilm was dislodged into the solution and log cell reduction was calculated by subtracting the cfu count for the test from the control biofilm. Bar graphs and tables were generated to represent the data and an agent causing a $\geq 4 \log_{10}$ reduction in cells was described as effective.

Figure 79 The effectiveness of 2% Tego 2000 against *Salmonella* cells in biofilm with one minute and five minutes exposure time

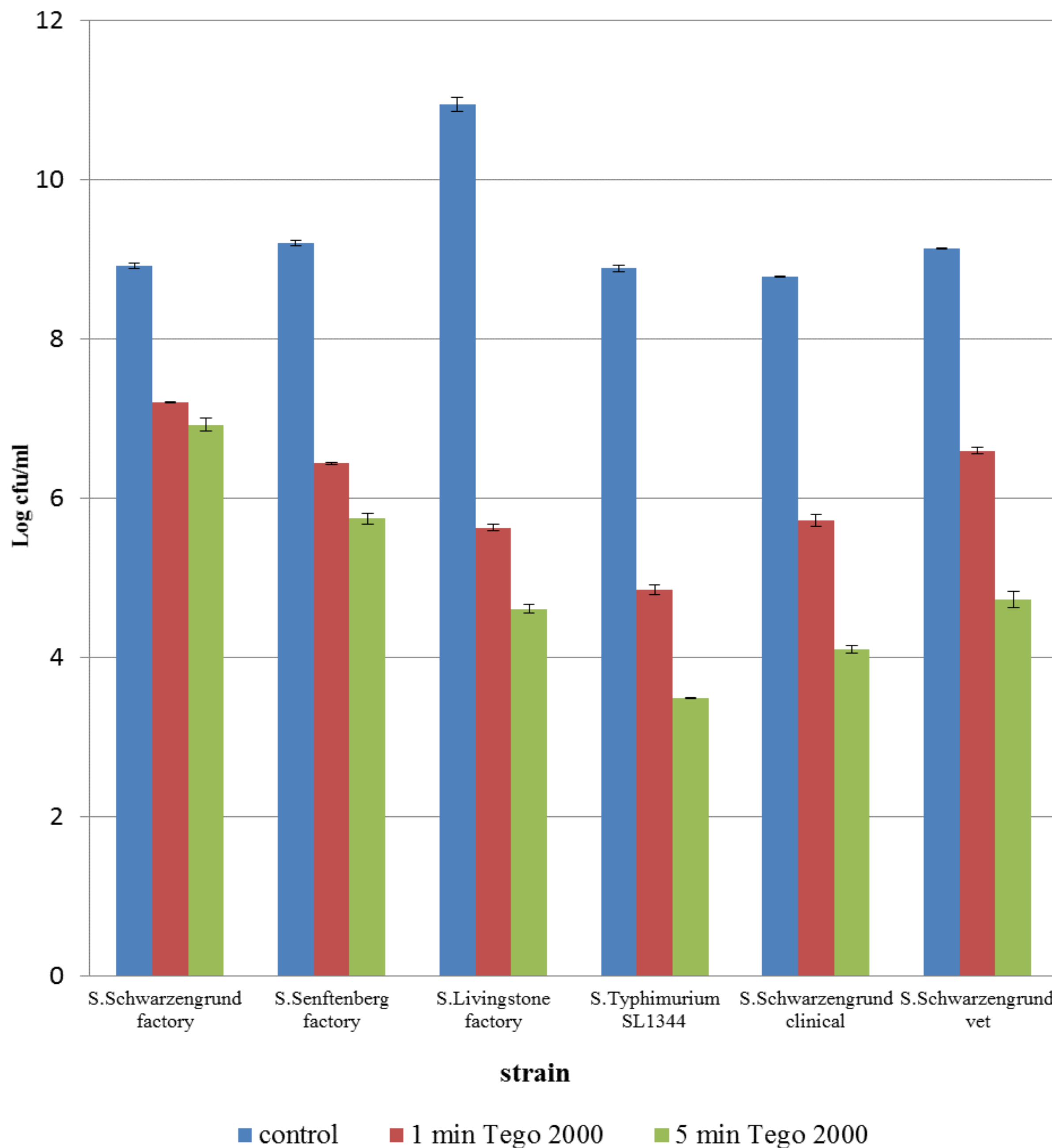


Figure 79 displays results from 1 minute and 5 minute contact time with Tego 2000 against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A reduction in cell number was observed for all strains tested with both exposure times, to calculate the $\log_{10}$ cell reduction, the cfu values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 13.

Table 13 The $\log_{10}$ reduction in cell number following 1minute and 5minute exposure to Tego 2000

biofilm strain	1 min Tego 2000	5 min Tego 2000
*S.*Schwarzengrund factory	1.721	1.995
*S.*Senftenberg factory	2.768	3.459
*S.*Livingstone factory	**5.314**	**6.332**
*S.*Typhimurium SL1344	**4.031**	**5.396**
*S.*Schwarzengrund clinical	3.063	**4.683**
*S.*Schwarzengrund vet	2.541	**4.412**

Table 13 reveals the calculated log10 cfu reduction following exposure at 1 min and 5 minutes. The cells highlighted in green show the strains that have achieved a ≥4 $\log_{10}$ reduction in cells attached to a surface after contact with the antibacterial agent. At 1 minute Tego 2000 was most effective against the factory isolate of *S.*Livingstone demonstrating a 5.3 $\log_{10}$ reduction and *S.*Typhimurium SL1344 (4.03 $\log_{10}$ reduction). The factory isolate of *S.*Schwarzengrund displayed the lowest reduction in cell number (1.72 log10 reduction). Following the increased 5 minute contact time a majority of the isolates displayed a ≥4 $\log_{10}$ reduction in cell number. With the exception of the factory isolate of *S.*Schwarzengrund which displayed a 1.995 log reduction and the factory isolate of S.Senftenberg which revealed a 3.46 log reduction in cell number.

Figure 80 The effectiveness of 2% Sodium Hypochlorite against *Salmonella* cells in biofilm with one minute and five minutes exposure time

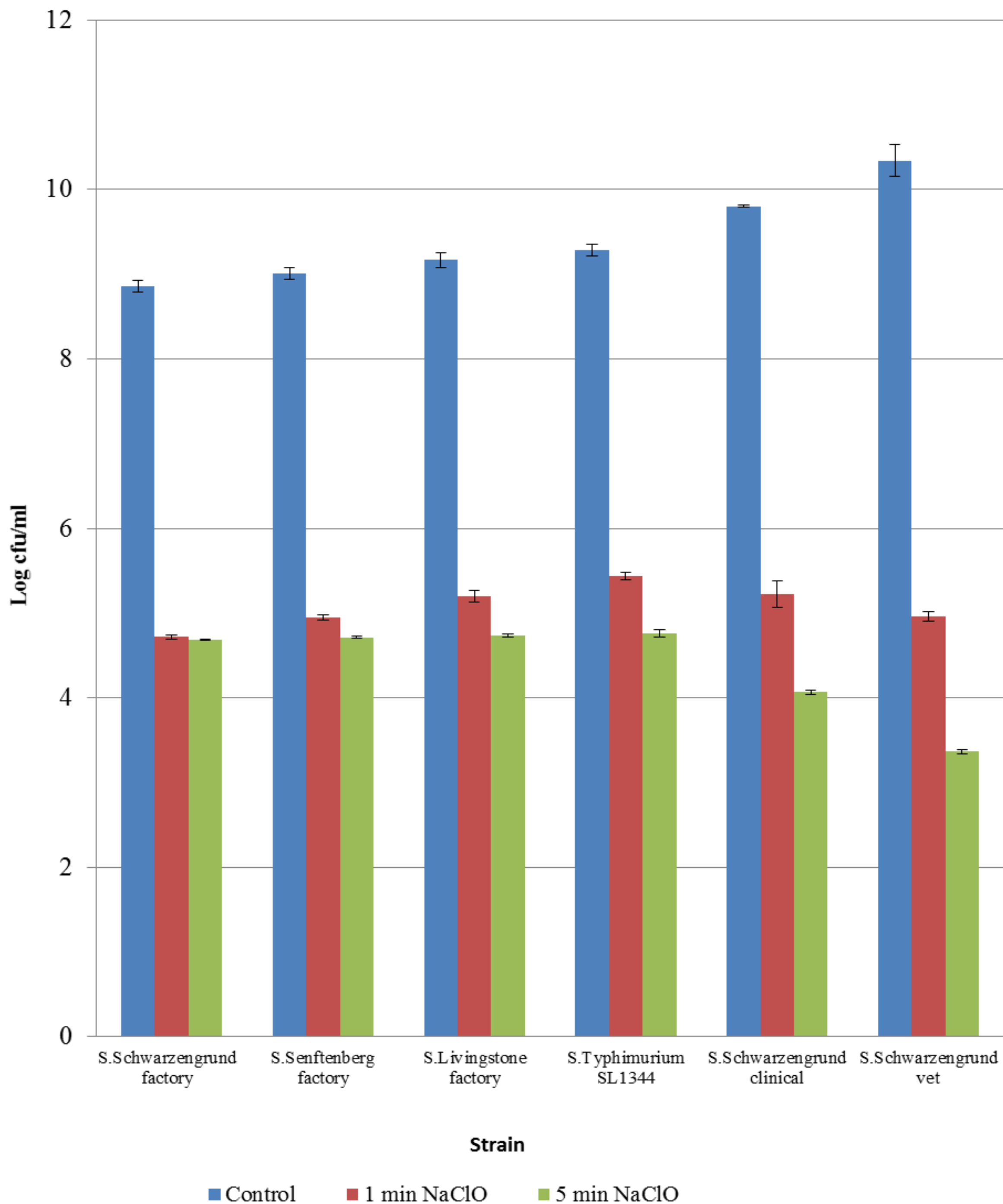


Figure 80 displays results from 1 minute and 5 minute contact time with 2% Sodium Hypochlorite against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A large reduction in cell number was observed for all strains tested with both exposure times, to calculate the log_{10} cell reduction, the cfu values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 14.

Table 14 The log_{10} reduction in cell number following 1minute and 5minute exposure to Sodium Hypochlorite

Control	**1 min NaClO**	**5 min NaClO**
S.Schwarzengrund factory	**4.135**	**4.166**
S.Senftenberg factory	**4.053**	**4.292**
S.Livingstone factory	3.968	**4.424**
S.Typhimurium SL1344	3.847	**4.522**
S.Schwarzengrund clinical (FSL S5-458)	**4.57**	**5.731**
S.Schwarzengrund vet	**5.378**	**6.97**

Table 14. reveals the calculated log_{10} cfu reduction following exposure at 1 min and 5 minutes. The cells highlighted in green show the strains that after 1 minute most isolates in the panel achieved a ≥4 log_{10} reduction in cells attached to a surface. The factory isolate of S.Livingstone exhibited a 3.968 log_{10} reduction and S.Typhimurium SL1344 achieved a 3.85 log_{10} reduction, both very close to the 4 log reduction in cell number. Following the increased 5 minute contact time all of the isolates displayed a ≥4 log_{10} reduction in cell number. With the veterinary isolate of *S*.Schwarzengrund revealing the highest log reduction (6.97 log_{10} cfu), closely followed by *S*.Schwarzengrund (American human clinical isolate FSL S5-458) which revealed a 5.73 log_{10} reduction in cell number.

Figure 81 The effectiveness of 2% Chlorhexidine against *Salmonella* cells in biofilm with one minute and five minutes exposure time

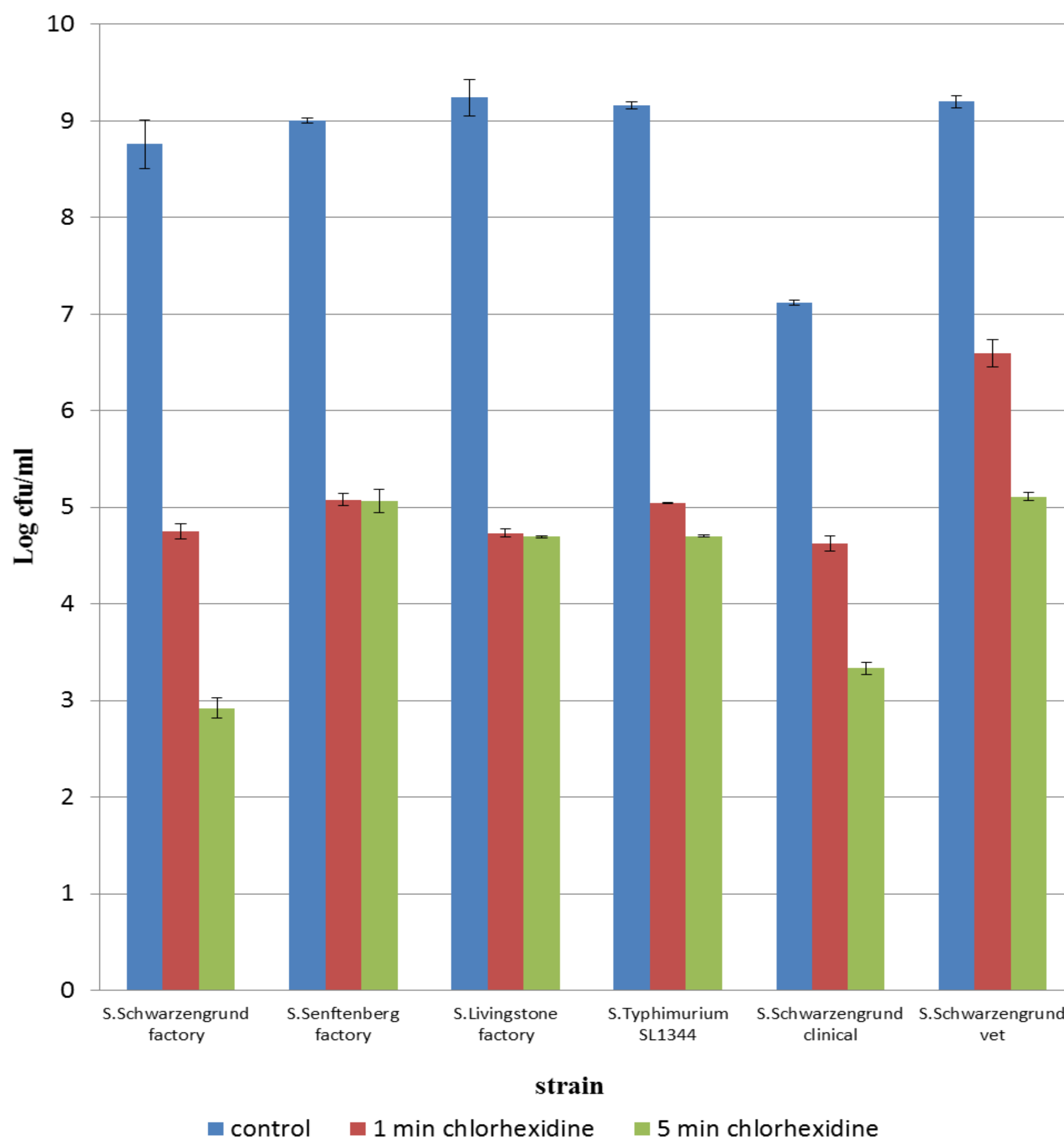


Figure 81 displays results from 1 minute and 5 minute contact time with 2% chlorhexidine against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A more variable reduction in cell number was observed across the strains tested with the two exposure times. To calculate the $\log_{10}$ cell reduction, the CFU values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 15.

Table 15 The log_{10} reduction in cell number following 1minute and 5minute exposure to 2% Chlorhexidine

strain	1 min chlorhexidine	5 min chlorhexidine
*S.*Schwarzengrund factory	**4.00**	**5.83**
*S.*Senftenberg factory	3.92	3.94
*S.*Livingstone factory	**4.50**	**4.54**
*S.*Typhimurium SL1344	**4.11**	**4.45**
*S.*Schwarzengrund clinical	2.49	3.78
*S.*Schwarzengrund vet	2.60	**4.08**

Table 15 reveals the calculated log_{10} cfu reduction following 2% Chlorhexidine exposure at 1 min and 5 minutes. The cells highlighted in green show the strains that after 1 minute the factory isolates of S.Schwarzengrund, *S.*Senftenberg and *S.*Typhimurium SL1344 achieved a ≥4 log_{10} reduction in cells attached to a surface. The factory isolate of *S.*Senftenberg closely followed with a 3.92 log_{10} reduction in cell number. However the *S.*Schwarzengrund (American human clinical isolate FSL S5-458) only revealed a 2.49 log_{10} reduction, alongside the veterinary isolate of *S.*Schwarzengrund which revealed a 2.60 log_{10} cell reduction. Following a 5 minute contact time a majority of the isolates displayed a ≥4 log_{10} reduction in cell number, the most sensitive being the factory isolate of *S.*Schwarzengrund (5.834 log_{10} cell reduction). The factory isolate of *S.*Senftenberg closely followed with a 3.94 log_{10} cell reduction and the *S.*Schwarzengrund (American human clinical isolate FSL S5-458) also revealed a 3.78 log_{10} reduction.

Figure 82 The effectiveness of 2% Benzalkonium chloride against *Salmonella* cells in biofilm with one minute and five minutes exposure time

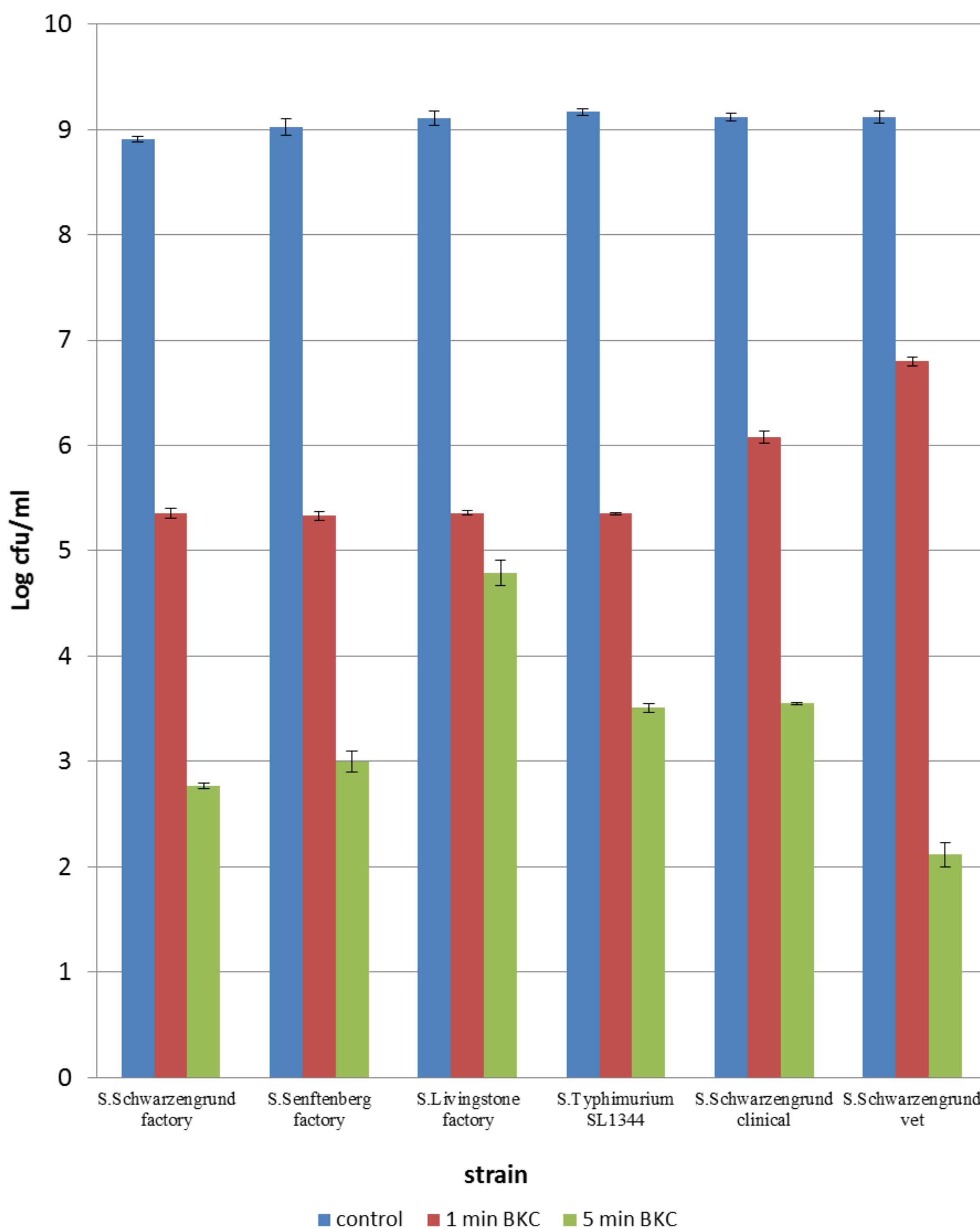


Figure 82 displays results from 1 minute and 5 minute contact time with 2% Benzalkonium chloride against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A more variable reduction in cell number was observed across the strains tested with the two exposure times. To calculate the log_{10} cell reduction, the CFU values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 16.

Table 16 The log_{10} reduction in cell number following 1minute and 5minute exposure to 2% Benzalkonium chloride

strain	1 min BKC	5 min BKC
*S.*Schwarzengrund factory	3.55	**6.13**
*S.*Senftenberg factory	3.69	**6.02**
*S.*Livingstone factory	3.75	**4.32**
*S.*Typhimurium SL1344	3.81	**5.66**
*S.*Schwarzengrund clinical	3.03	**5.56**
*S.*Schwarzengrund vet	2.31	**7.00**

Table 16 reveals the calculated log_{10} cfu reduction following BKC exposure at 1 min and 5 minutes. Interestingly, all though 1 minute exposure reveals reduction in cell number, none of the isolates achieved a ≥ 4 log_{10} reduction in cells attached to a surface. However following a 5 minute contact time all the isolates displayed a ≥ 4 log_{10} reduction in cell number, the most effective being against the veterinary isolate of *S.*Schwarzengrund, which exhibited a 7 log_{10} cell reduction. This was followed by the factory isolate of *S.*Schwarzengrund which displayed a 6.13 log_{10} reduction and the factory isolate of *S.*Senftenberg which revealed a 6.02 log_{10} reduction.

Figure 83 The effectiveness of 2% Virkon against *Salmonella* cells in biofilm with one minute and five minutes exposure time

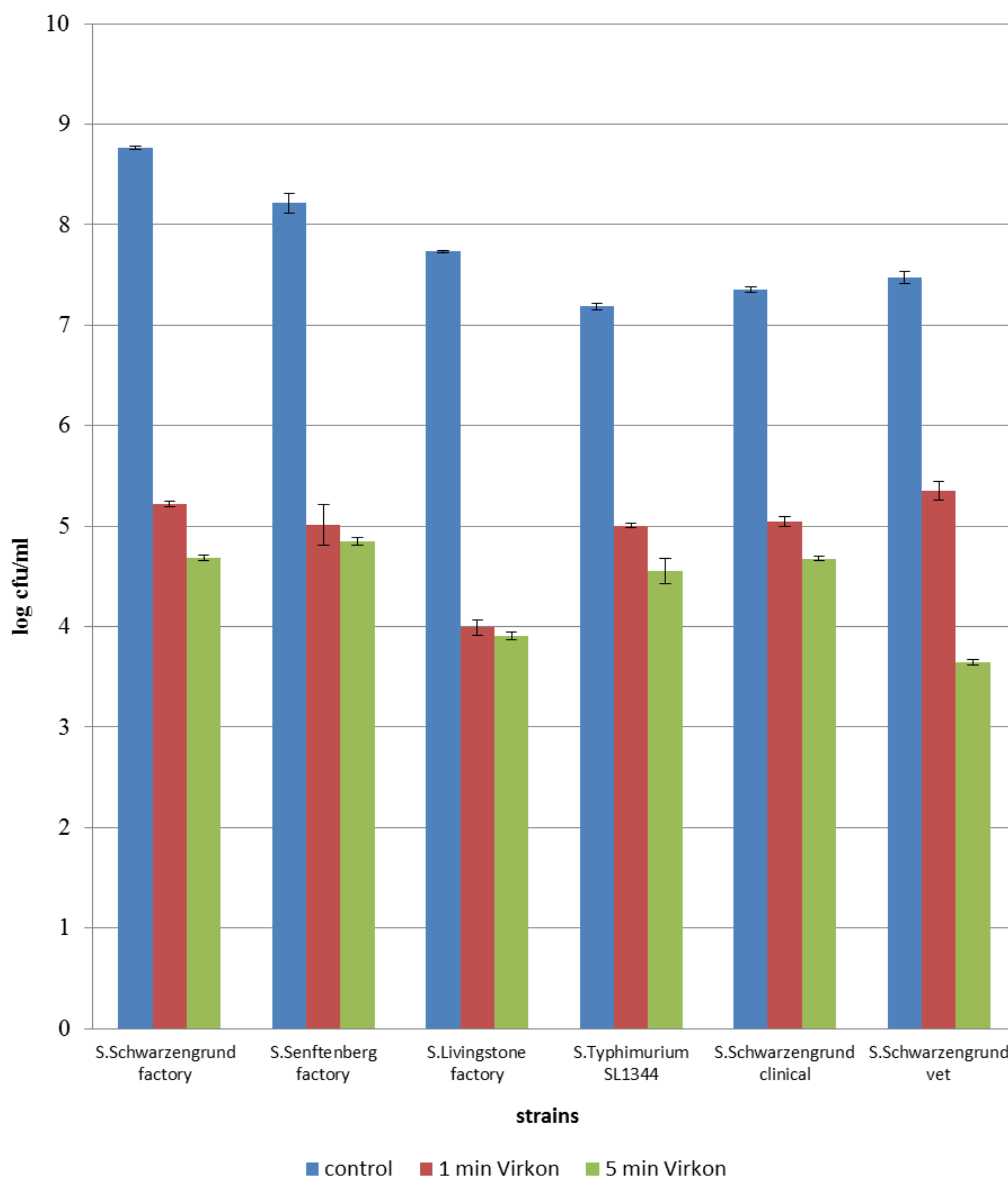


Figure 83 displays results from 1 minute and 5 minute contact time with 2% Virkon against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A reduction in cell number was observed across the strains tested with the two exposure times. To calculate the $\log_{10}$ reduction, the CFU values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 17.

Table 17 The log_{10} reduction in cell number following 1minute and 5minute exposure to 2% Virkon

strain	1 min Virkon	5 min Virkon
S.Schwarzengrund factory	3.547934793	4.081792203
S.Senftenberg factory	3.203478751	3.367002493
S.Livingstone factory	3.739875788	3.827946758
S.Typhimurium SL1344	2.181803475	2.633545284
S.Schwarzengrund clinical	2.309913048	2.68124097
S.Schwarzengrund vet	2.122973185	3.830755448

Table 17 reveals the calculated log_{10} cfu reduction following Virkon exposure at 1 min and 5 minutes. Overall, Virkon was least effective against *Salmonella* biofilms, with none of the isolates achieving a ≥4 log_{10} reduction in cells after 1 minute exposure. However following a 5 minute contact time although all the log reductions increased, only the factory isolate of *S*.Schwarzengrund displayed a 4.08 log_{10} reduction, closely followed by the factory isolate of *S*.Livingstone which revealed a 3.83 log_{10} cell reduction and the veterinary isolate of *S*.Schwarzengrund which revealed a 3.83 log_{10} cell reduction.

Figure 84 The effectiveness of 2% Peracetic acid against *Salmonella* cells in biofilm with one minute and five minutes exposure time

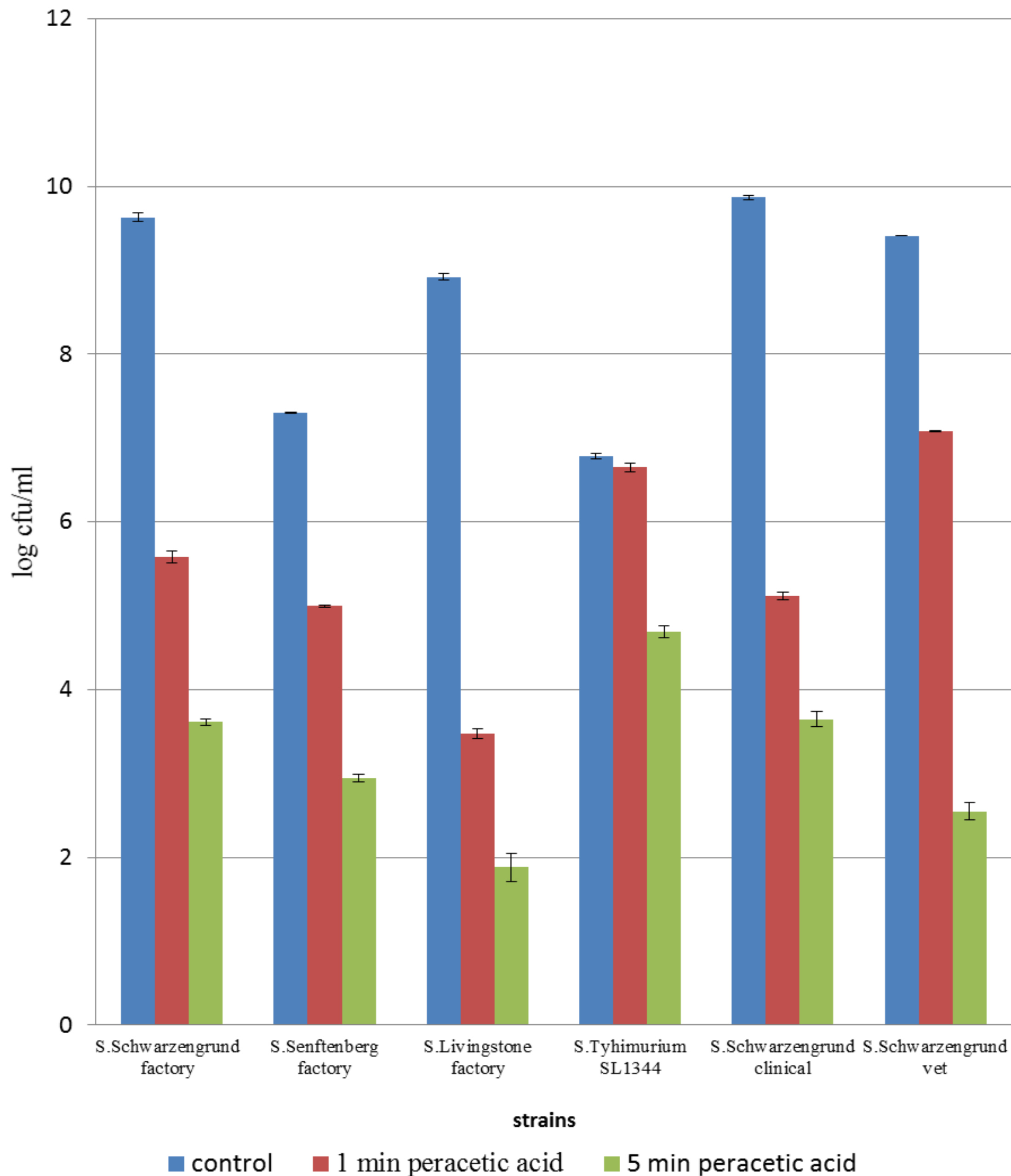


Figure 84 displays results from 1 minute and 5 minute contact time with 2% peracetic acid against 48hour mature *Salmonella* biofilms. The error bars represent standard deviation. A reduction in cell number was observed across the strains tested with the two exposure times. However increased exposure time was important for greater cell reduction. To calculate the log reduction, the log_{10} cfu values obtained following one minute and five minute contact time were subtracted from the control biofilm value and the values displayed in Table 18.

Table 18 The $\log_{10}$ reduction in cell number following 1minute and 5minute exposure to 2% peracetic acid

strain	1 min peracaetic acid	5 min peracaetic acid
*S.*Schwarzengrund factory	**4.04**	**6.02**
*S.*Senftenberg factory	2.30	**4.36**
*S.*Livingstone factory	**5.44**	**7.04**
*S.*Typhimurium SL1344	0.133	2.09
*S.*Schwarzengrund clinical	**4.75**	**6.21**
*S.*Schwarzengrund vet	2.33	**6.86**

Table 18 reveals the calculated $\log_{10}$ cfu reduction following peracetic acid at 1 min and 5 minutes exposure. After 1 minute exposure all strains revealed a reduction in cell number, however only the factory and clinical isolates of *S.*Schwarzengrund and the factory isolate of *S.*Livingstone revealed a ≥4 $\log_{10}$ reduction in cell number. Following a 5 minute contact time all the isolates displayed a ≥4 $\log_{10}$ reduction in cell number, with the exception of *S.*Typhimurium SL1344 revealing a 2.092 $\log_{10}$ cell reduction. Peracetic acid was most effective against the factory isolate of *S.*Livingstone which exhibited a 7 $\log_{10}$ cell reduction. This was followed by the veterinary isolate of *S.*Schwarzengrund which displayed a 6.86 $\log_{10}$ cell reduction. Although the increased exposure resulted in a decrease in cell count for *S.*Typhimurium only a 2.092 $\log_{10}$ cell reduction was observed.

6.4 Disinfection penetration through biofilms on membrane

The complete penetration of the disinfectants through biofilms grown on a polycarbonate membrane was tested using an alternative method. Biofilms were transferred onto disinfectant containing agar plates and a concentration disk moistened in saline was placed on top of the biofilm. A disinfectant capture disc was placed on top of the biofilm and the assay was incubated for 18-24 hours at 37°C. Following incubation, the disinfectant capture disc discs were placed on a nutrient agar plate that had been inoculated with 10^4 cfu/ml and incubated overnight at 37°C and the zone of inhibition measured; this was used to determine the concentration of active disinfectant that had penetrated through the biofilm. The initial and test biofilm load was also determined and recorded.

** this experiment was performed with all the disinfectants however the positive control **only** revealed zones of inhibition with Tego 2000.

Figure 85 The effect of Tego 2000 on biofilm load following 24 hours incubation

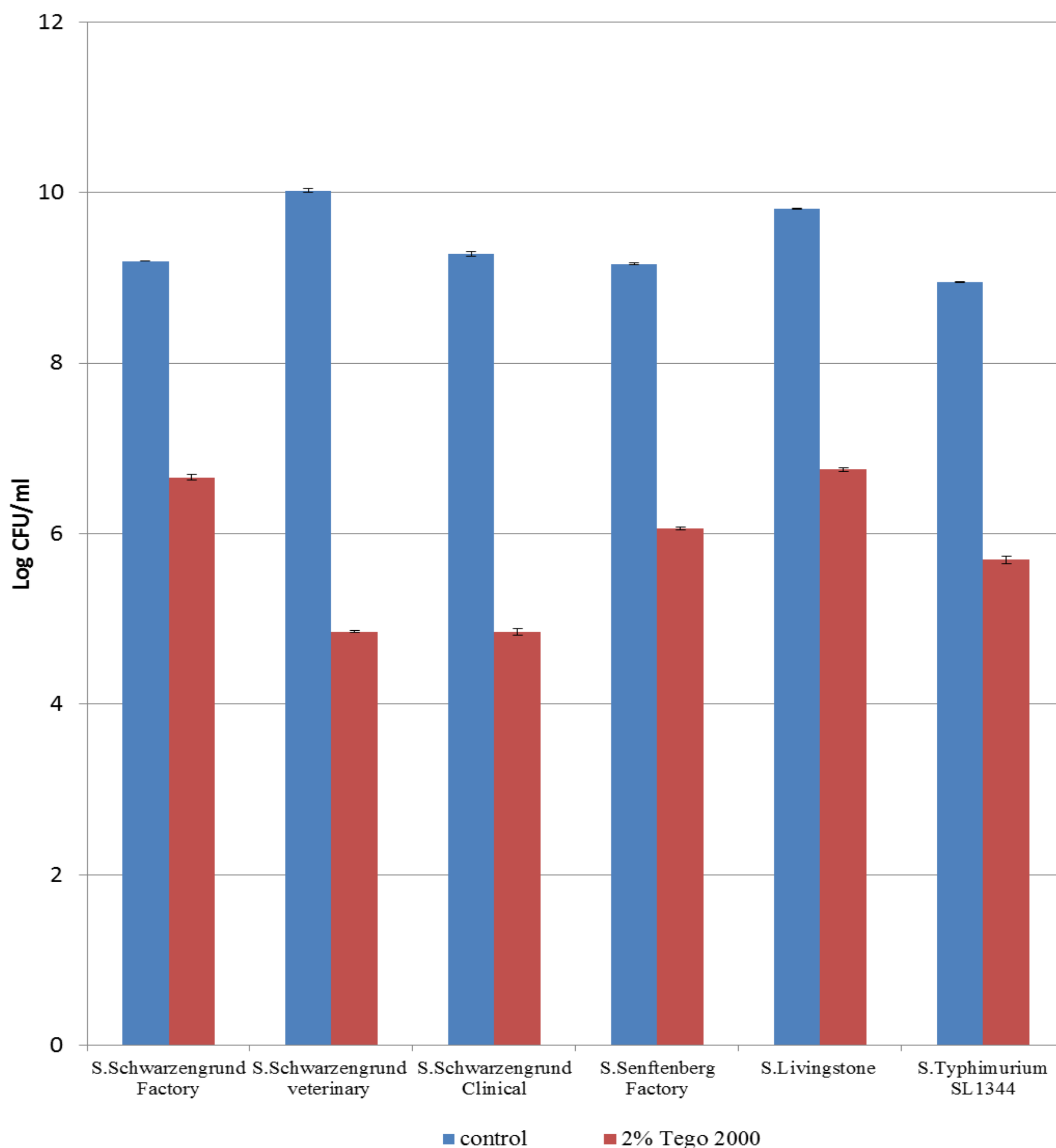


Figure 85 displays results from the disinfectant penetration assay, the blue bars represent the untreated biofilm and the red bars represent the recovered cell count following 24 hour incubation on agar containing 2% Tego 2000. The error bars indicate standard deviation. Results reveal a clear reduction in cell count across all isolates showing that Tego 2000 can penetrate through a 48hour *Salmonella* biofilm with 24hours contact time. Tego 2000 was most effective against the veterinary isolate of *S.*Schwarzengrund (7.17 $\log_{10}$ cell reduction) and the factory isolate of S.Livingstone (7.11 $\log_{10}$ cell reduction) followed by the clinical isolate of *S.*Schwarzengrund (4.42 $\log_{10}$ cell reduction). The remainder of the isolates revealed an average 6 $\log_{10}$ cell reduction. Tego 2000 demonstrated a 5.25 $\log_{10}$ cell reduction against S.Typhimurium SL1344.

Figure 86 the penetration of Tego 2000 through a biofilm grown on membranes

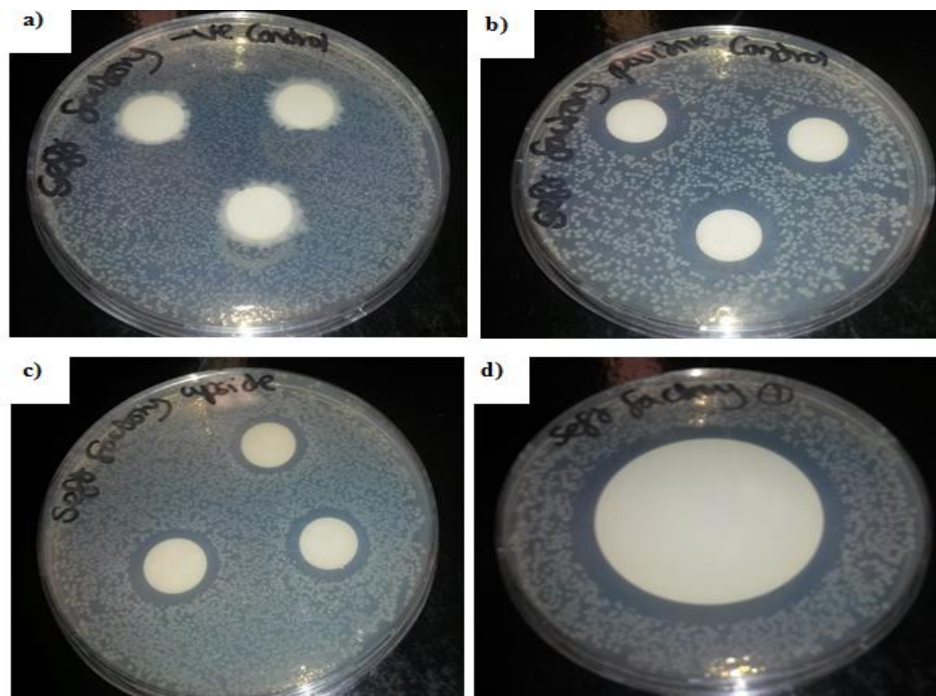


Figure 86 a) discs taken from negative control plate with no disinfectant added to the agar, the plate shows growth up to the disc and no inhibition. b) discs taken from positive control plate, whereby the membrane had no biofilm and 2% of Tego 2000 was added to the agar, this shows the disinfectant can penetrate through a blank membrane and is represented via the clear zones around the discs where growth has been inhibited c) Discs taken from test plate with a 48 hour biofilm incubated on agar containing 2% Tego 2000, after 24 hours clear zones can be seen where the disinfectant has penetrated through the biofilm and the blank membrane to the disc and inhibited growth around the disc d) a clear zone around a membrane placed on top of the biofilm, showing the disinfectant is penetrating through the membrane.

Table 19 Zone sizes demonstrating ability of Tego 2000 to penetrate through a biofilm following 24 hours incubation

	Zone of inhibition around disc (cm)		
Strains	2% Tego 2000	Positive control no biofilm	Negative control
***S.*Schwarzengrund Factory**	0.35	0.76	No Zone
S.Schwarzengrund veterinary	0.366	0.73	No Zone
***S.*Schwarzengrund Clinical**	0.3	0.73	No Zone
S.Senftenberg Factory	0.65	0.76	No Zone
S.Livingstone	0.43	0.76	No Zone

Table 19 reveals that Tego 2000 is capable of penetrating through the membrane to the top disc, the largest zone was observed for the factory isolate of *S.*Senftenberg. The remainder of the isolates exhibited zones between 0.3 and 0.43cm. However it is important to note that these results were obtained after 24 hours incubation, suggesting that a considerable contact time is required for effect.

6.5 Discussion

The Minimum Inhibitory Concentration (MIC) of the disinfectants was determined using the micro broth dilution method (WHO, 2010). It can be defined as the lowest concentration of the antimicrobial agent required to inhibit microbial growth and it is usually only a small fraction of the recommended concentration. Establishing the MIC provides an indication of the efficacy of the disinfectant against isolates in their planktonic state.

The results described in Table 13 indicate that all the antimicrobial agents were bactericidal and inhibited visible bacterial growth, although the concentrations varied. A majority of the individual MIC values for the disinfecting agents were similar across the panel of isolates with a few exceptions. The MIC values of Sodium Hypochlorite for the factory isolates of *S.*Kedougou and *S.*Montevideo (0.78%) were almost twice that in comparison to the other isolates (0.38%). A concentration of 0.001% Sorgene and 1% Virkon were sufficient to inhibit growth across all isolates. Low quantities of Tego 2000, Biosan (< 0.003%) and Peracaetic acid (<0.09%) were sufficient in causing a reduction for a majority of the isolates. For BKC all *Salmonella* isolates had a MIC of <0.03% with the exception of the factory isolate of *S.*Kedougou which had an MIC of 0.125%. The lowest MIC value for chlorhexidine was observed for the clinical isolate of *S.*Senftenberg (0.0156%) however highest was observed for the factory and veterinary isolates of *S.*Senftenberg (0.25%) and the MIC values for the remainder of the isolates was 0.125%.

Although the Minimum Bactericidal Concentration (MBC) was not determined experimentally, the fact that the MIC values were lower than the manufacturers recommended concentrations suggests with some confidence it can be expected these chemicals can kill the organisms if applied at the correct concentration with the correct contact time.

It is fundamental that disinfectants are prepared with the correct dilutions, as failure of a disinfectant can be attributed to use of disinfectant solutions being too dilute. The concentration exponent, η, is defined as the relationship between dilution and activity of a biocide, it is calculated by determining the time to killing of a specified proportion of the population at a particular concentration (Ioannou *et al.*, 2007). Incorporation of the concentration exponents of chemicals is valuable as a large

difference is visualised across chemicals. For example the concentration exponent of chlorhexidine is 2; therefore a 3 fold dilution will result in a 3^6 reduction in disinfection activity (Ascenzi, 1995). Disinfectants with larger concentration exponents or dilution coefficient rapidly lose activity when diluted. Unlike in the clinical setting, the MIC of chemicals in a factory environment are likely to also be compromised by organic inactivation and other neutralising factors, therefore when biocides are used in the industry it is important to take ensure that the concentration is sufficiently high that any neutralisation that may occur is accounted for.

However in reality cells found in the food manufacturing environment are unlikely to exist in the planktonic state so although growth is inhibited, the agents are not as effective against *Salmonella* cells attached to a surface in a biofilm (Mah and O'Toole, 2001; Spoering and Lewis, 2001; Moretro *et al.*, 2009b).

These data supports the findings of Moretro *et al.* (2003) who also reported that an MIC of 0.01-0.005% BKC was effective at eliminating bacterial growth in a suspension test, but the same concentration was not effective in eliminating cells in biofilm (Corcoran *et al.*, 2014). Furthermore, Chuanchuen *et al.* (2008) investigated the susceptibility of *Salmonella enterica* isolates from poultry and swine to disinfectants including both BKC and Chlorhexidine. Results also revealed there were few variations in MIC values across the disinfectants, indicating that the isolates either had not acquired or only a limited degree of developed resistance to the disinfectants tested (Chuanchuen *et al.*, 2008).

The six agents tested against 48 hour biofilms were effective to varying degrees. However none of the disinfectants were effective in fully eliminating all cells in biofilm. The research presented in this chapter indicates that Sodium hypochlorite was effective against planktonic cells, inhibiting cell growth using a suspension test method and was very effective at 2% with both contact times against cells in a biofilm. All factory strains revealed a ≥4 log_{10} reduction in cells attached to a surface following five minutes exposure, the highest reduction was observed for the veterinary isolate of *S.*Schwarzengrund, which revealed a 6.97 log_{10} reduction in cell number, closely followed by *S.*Schwarzengrund (American human clinical isolate FSL S5-458) revealing a 5.73 log_{10} reduction in cell number. With the contact times tested, this reduction was as anticipated as the recommended is 5.25-6% and perhaps with

increased exposure/concentration sodium hypochlorite may result in complete elimination of the cells.

In line with our data,Buckingham-Meyer *et al.* (2007) also revealed that sodium hypochlorite exposure against static biofilm methods and biofilms grown on dried surfaces resulted in a ~4 log_{10} reduction in cells recovered from the surface, although contact with same disinfectant using the CBR method resulted in a ~1-2 log_{10} reduction in the number of cells (Buckingham-Meyer *et al.*, 2007),thus emphasising that variability in results may be seen even with the same strains depending on the method used to grow biofilms. Nguyen & Yuk (2013) revealed a ≥4 log_{10} reduction with 0.005% sodium chlorite, against biofilms grown on acrylic surfaces using a petri dish with bacterial suspension, for 48 hours. Against biofilms grown on stainless steel for 24 hours, their research showed >8 log_{10} reduction in cell number on both stainless steel and acrylic surfaces (Nguyen and Yuk, 2013). Furthermore Ramesh *et al.* (2002) also reported a high cell reduction for cells in biofilm with sodium hypochlorite. Biofilms were grown in a static micro titre plate on stainless steel coupons and reported that a 0.025% concentration of sodium hypochlorite was sufficient in causing a 6.26 log_{10} cell reduction following 1 minute contact time with *Salmonella* biofilms grown for 96 hours. A further 2 minutes resulted in <0 cells detected recovered from the surface. Both these concentrations are considerably lower implicating that results may be serotype and biofilm method dependant.

Tondo *et al.* (2010) investigated *Salmonella* attachment to polyyethene and the effect of exposure to disinfectants for 15, 30 and 60 minutes, these conditions were set to mimic how food may contact contaminated surfaces in the processing environment. Results revealed a 2-3 log_{10} reduction in *Salmonella* cells recovered from polyethene surfaces after contact with sodium hypochlorite even with increased exposure.

However other research, has suggested that viable cells were recovered from biofilms grown for 48hours even after 90 minutes of contact time (Corcoran *et al.*, 2014). Moretro *et al.* (2009b) studied biofilm formation of *S.* Agona and *S.* Typhimurium on glass slides with 48 hour incubation. Their findings suggested only a 0.5-1 $\log_{10}$ reduction in the cells after 5 minutes contact time with sodium hypochlorite. It is clear that variability in recovered cell number is likely to be attributed to by the method and surface used for biofilm formation; both Moretro *et al.* (2009b) and Corcoran *et al.* (2014) used CBR to produce biofilms. This method involves growing biofilms on coupons in a CDC biofilm reactor and was first described by Donlan (2002), it allows 24 removable of biofilm coupons for sampling and analysing the biofilm.

Benzylkonium chloride (2%) achieved an average of 3.32 $\log_{10}$ cell reduction after 1 minute and all isolates displayed a ≥4 $\log_{10}$ reduction following 5 minutes exposure, this is a relatively high concentration considering the recommended is 0.75%. Other studies have also reported the efficacy of BKC being lower in comparison to other agents and many have reported longer exposure times are needed to achieve full effect. Corcoran *et al.* (2014), indicated a 1.5 $\log_{10}$ reduction after a lengthy 90 minutes against a 48 hour biofilm using the recommended concentration. Wong *et al.* (2010a) also found that only sodium hypochlorite successfully eliminated all cells at the recommended concentration, whereas in order to eradicate all cells using BKC the lower concentrations were ineffective and a 1.5% concentration was used. Vestby *et al.* (2009b) reported 0.02% Benzalkonium chloride applied for 5 minutes against a 48 hour *Salmonella* biofilm revealed a in a 1-2 $\log_{10}$ reduction in the number of cells recovered from the surface.

Peracetic acid (PAA) is a peroxygen sanitiser and is known as an effective biocide due to its ability to act even in the presence of organic load. The results with percaetic acid varied, the average MIC value was 0.04% to 0.09% and the lowest 0.0015%. After 1 minute exposure only the factory and veterinary isolates of *S.*Schwarzengrund and the factory isolate of *S.*Livingstone biofilms revealed a ≥4 $\log_{10}$ reduction in cell number. Following 5 minutes contact time with peracetic acid all the isolates in biofilm displayed a ≥4 $\log_{10}$ reduction in cell number, with the exception of *S.*Typhimurium SL1344 revealing a 2.092 $\log_{10}$ cell reduction. Similarly, Bauermeister *et al.* (2008) found that concentrations as low as 0.0025% of PAA were effective in decreasing *Salmonella* spp in the planktonic state and concentrations of 0.015% may extend product shelf-life, which have applications in poultry chillers. Tondo *et al.* (2010),

found that the effect of PAA varied with surface, with polyethylene a 2.4 to 3.3 *Salmonella* log_{10} reduction was observed, whereas a higher reduction (2.5 to 4.0 log_{10} cell reduction) was observed for stainless steel (Tondo *et al.*, 2010).

Many commercially available biocide formulations used in the food industry contain CHX as the active ingredient (Block, 2001; Condell *et al.*, 2012). Results from the current study reveal that the MIC of Chlorhexidine (CHX) against planktonic cells varied across the *Salmonella* isolates and ranged between 0.0156 to 0.25%. In a biofilm, a majority of the isolates resulted in a ≥4 log_{10} cell reduction with both 1 minute and 5 minutes exposure to 2% CHX. However .following one minutes exposure, *S*.Schwarzengrund (American human clinical isolate FSL S5-458) only revealed a 2.49 log_{10} cell reduction, alongside the veterinary isolate of *S*.Schwarzengrund which revealed a 2.60 log_{10} cell reduction. However an increased contact time of five minutes resulted in a higher revealed a 3.78 log_{10} cell reduction for the veterinary isolate and a 4 log_{10} cell reduction for the clinical isolate, emphasising the importance of following contact times stated on cleaning protocols.

Block (2001), also revealed a difference in MIC values across serotypes, with concentrations ranging from 4-16mg/L across different serotypes of *Salmonella.* Furthermore varying results were seen across different serotypes of *Salmonella* when 0.05% CHX was added to planktonic cells of *Salmonella*, with one minute contact time revealing a range 2.9-4.0 log_{10} cell reduction (Block, 2001). Condell *et al.* (2012), revealed that CHX was effective against *Salmonella* isolates in the planktonic state and exposing *Salmonella* isolates to sub-inhibitory concentrations of chlorhexidine (equivalent to 0.25 times the MIC) caused a 25 to 50 fold increase in the MIC. Importantly it was also noted that the laboratory type strains were slower to acquire tolerance compared to strains recovered from the environment. This could be linked to the fact that the factory strains have been previously exposed to selective conditions. Importantly this study reiterates that sub lethal exposure to CHX and BKC can lead to the development of tolerant isolates (Condell *et al.*, 2012).

Virkon is a commercially available disinfectant which is a combination of potassium permonosulfate and sodium chloride. A concentration of 1% Virkon is a recommended and frequently used as a disinfectant in the industrial setting (Gehan *et al.*, 2009), however although effective in decreasing cell counts against *Salmonella* in the planktonic form, pilot studies showed no reduction against *Salmonella* cells in biofilms therefore the concentration was doubled to 2%. The results in this chapter

indicate that Virkon was the least effective agent against *Salmonella* biofilms, with none of the isolates achieving a ≥4 $\log_{10}$ reduction in cells after 1 minute exposure. However following a 5 minute contact time although all the log reduction values increased, only the factory isolate of *S*.Schwarzengrund displayed a 4.08 $\log_{10}$ reduction, closely followed by the factory isolate of *S*.Livingstone and the veterinary isolate of *S*.Schwarzengrund revealed a 3.82 $\log_{10}$ cell reduction. Moretro *et al.* (2009b), tested the efficacy of Virkon S against 2 days-old biofilms of *S*. Agona and *S*. Senftenberg on stainless steel and showed a >4 $\log_{10}$ reduction in the number of *Salmonella* cells following 5 minutes contact time. Again this reiterates that differences in efficacy of disinfectants vary depending on the surface used for biofilm attachment. Gehan *et al.* (2009) evaluated the efficacy of five disinfectants including Virkon against a panel of strains isolated from poultry facilities and also found 1% Virkon was infective against *Salmonella* after 1,10,30 and 60 minutes contact time. These results indicate the importance of cleaning surfaces to remove organic matter prior to disinfection (Gehan *et al.*, 2009).

Tego 2000 is an amphoteric and cationic disinfectant routinely used in the food industry and claims to be effective against both Gram-positive and Gram-negative bacteria at concentrations of 0.5-2%. Against planktonic cells Tego 2000 was effective at a concentration of 0.003%. Following 1 minute contact in 2% Tego 2000 was most effective against the biofilm of factory isolate of *S*.Livingstone demonstrating a 5.3 $\log_{10}$ reduction however the factory isolate of *S*.Schwarzengrund displayed the lowest reduction in cell number (1.72 $\log_{10}$ reduction). Following the increased 5 minute contact time a majority of the isolates displayed a ≥4 $\log_{10}$ reduction in cell number, except the factory isolate of *S*.Schwarzengrund which displayed a 1.995 $\log_{10}$ reduction and the factory isolate of *S*.Senftenberg which revealed a 3.46 $\log_{10}$ reduction in cell number. The membrane penetration assay results showed that of the six agents tested with increased exposure time of 24 hours, only Tego 2000 was able to penetrate through the membrane and cause a significant cell reduction (5-7$\log_{10}$ cfu/ml). The remainder of the chemical agents were unable to penetrate through biofilms on membranes or could possibly be inactivated by the medium. Tego 2000 also penetrated through to the disinfectant capture disc and produced zones of inhibition.

This is important as there is large variability in the efficacy of Tego 2000 with contact time against factory isolates which could have major implications in the food industry. It would depend on how Tego 2000 would be applied in the food industry, in some plants (i.e dairy) biocides are rinsed away whereas in other industries, disinfectants are not washed away and in these situation 24 hours contact time would be of significance as over time exposure to sub lethal concentrations and build-up of chemical residuals can lead to resistance. Braoudaki & Hilton (2004), reported the MIC of BKC against *S.* Virchow increased from 4 to 256µg/ml as adaptation progressed. This study also revealed cross resistance was caused by repeated exposure to sub inhibitory concentrations of antimicrobial agents.

In the literature we were unable to find any studies conducted on *Salmonella* for comparison however data have been published on the efficacy with other organisms. Th. Goldschmidt AG studied the antimicrobial activity of Tego 2000 on *Staphylococcus aureus* and *Pseudomonas aeruginosa* and reported a concentration of 0.1% achieved a 4 log_{10} reduction after 5 minutes and a concentration of 0.25% lead to no significant difference in activity after 5 minutes even with increased contact time of 60 minutes (Block, 2001; Eissa *et al.*, 2014).

Other studies have compared the efficacy of Tego 2000 and Tego 51 with 70% Isopropyl alcohol on stainless steel, wall, floor, and curtain materials which are commonly found in factory plants. Results indicated that 1 and 5 minutes exposure against *Bacillus subtilis, Pseudomonas aeruginosa* and *Candida albicans,* a 3 log reduction was achieved and after 5 minutes of contact no cells were detected with all the agents tested across the four surfaces tested (Eissa *et al.*, 2014).

The results presented in this chapter indicated there are many methods available for both growing biofilms and testing the efficacy of disinfectants against biofilms. It is important to note that the method employed to grow biofilms and the surfaces being tested are key in the interpretation of the efficacy of biocides. Currently previous research indicates a lack of standardization in terms of methodology used for biofilm growth making it difficult to draw significant comparisons between our results and published literature. There are clear differences amongst results from biofilms grown statically in micro titre plates and those via CBR, even with the same set of strains. Furthermore variability is seen across test microbes and temperature/pH combinations (Al-Fattani and Douglas, 2004; Stepanovic *et al.*, 2004; Moretro *et al.*, 2009a; Corcoran *et al.*, 2014).

The biofilms used to test the efficacy of disinfectants were grown in full strength medium, results from the previous chapter and findings by Stepanovic *et al.* (2004) showed that *Salmonella* isolates grew significantly stronger biofilms in 1/20 diluted TSB media, therefore further investigation is required to show the effect of disinfectants against isolates grown in diluted media. Furthermore other work has indicated that *Salmonella* biofilm developed over 168 hours were less susceptible to the effect of disinfectants products compared to biofilms grown over 48 hours (Corcoran *et al.*, 2014).

Other studies have also stated the importance of contact times when applying chemical agents to biofilms (Wong *et al.*, 2010b). The results from the current study indicated that a minimal contact time of one minute was sufficient in reducing viable cell counts, however not all the agents tested resulted in a $\geq 4 \log_{10}$ reduction in cell number across the serotypes. With a contact time of five minutes a majority of the isolates achieved a $\geq 4 \log_{10}$ cell reduction however none of the disinfectants achieved complete eradication of the biofilm. Thus, sufficient contact times need to be implemented in cleaning SOP's to ensure effective removal of *Salmonella* cells, as shorter contact times do not lead to the desired cell reduction.

Further investigation, testing the acquired resistance through repeated exposure may be of importance. Some of the isolates will already have been pre-exposed to many of the chemical agents tested in the current study, which could explain the lower cell reduction observed for Tego 2000 and Virkon, both of these disinfectants are commonly used in the food industry and as mentioned above, studies have shown that environmental strains exposed to repeated sub lethal concentrations of disinfectants can lead to resistance (Condell *et al.*, 2012). To prevent resistance to agents it is important that plants frequently rotate the disinfectants used.

6.6 Conclusion

Understanding the relationship between *Salmonella* biofilms and disinfectants is crucial in the control of bacterial contamination for the food industry. Results in this chapter showed that planktonic cells were more susceptible to disinfectants than *Salmonella* cells in biofilm. Secondly although all the disinfectants tested were successful in reducing bacterial load none completely eradicated cells in biofilm. Lastly applying disinfectants for the correct contact time is essential when removing cells in biofilm as overall a one minute contact time did not achieve the desired cell reduction.

7 Chapter 7 Addressing the global genomic differences across isolates

7.1 Introduction

Addressing concerns relating to food safety are important especially in the current climate where import and export of foods is at a global level. Despite strategies already implemented to prevent the spread of pathogens, *Salmonella* is a leading cause of foodborne illness with *S.*Enteritidis and *S.*Typhimurium being the two most commonly isolated serotypes (Herikstad *et al.*, 2002). However recent outbreaks linked to food factory contamination have been caused by more rare serotypes such as *S.*Schwarzengrund, *S.*Montevideo and *S.*Livingstone (Finn *et al.*, 2013a). It is essential to address the underlying factors driving these serotypes to contaminate products in the factory environment.

In the investigations discussed in previous chapters, the factory isolates as a group have not shown any enhanced phenotypic, morphological or survival advantages that may lead to their persistence. However there may be differences at the genomic level which are not immediately expressed as measurable traits. Generally DNA is similar across a bacterial species, however there are places where there are differences which may encode for genes required for survival in stressful conditions such as heat resistance or starvation, and these differences act as genetic signatures that can be detected and examined producing a unique bacterial fingerprint.

Whole-genome sequencing (WGS) allows the examination of complete or nearly complete genomes of bacterial isolates and can theoretically reveal parts of a genome that may only differ at a single nucleotide level. Whole genome sequencing of bacteria is a multistep process that requires extracted DNA to be prepared in a library, sequenced, aligned/assembled and interpreted (Van El *et al.*, 2013; Salipante *et al.*, 2015). The principles of automated WGS systems are based on electrophoresis in that extracted DNA is loaded into a gel in automated sequencers and depends on the movement of DNA through the gel.

DNA is tagged with fluorescent dyes that can then be read by automated sequencers. The fluorescent tags correspond to the four different DNA bases (ATCG). In the automated sequencer a laser causes the dyes to fluoresce and a detector reads the colour of fluorescence and enables the software programme to decipher the

corresponding base. Each sequence grows one base at a time and at ~ 500 bases, this sequence is known as a read. This information is then stored on the system and following completion the multiple DNA reads of raw sequence are aligned and assembled by comparison with a reference sequence (GNN, 2004).

De novo sequencing refers to sequencing a genome for the first time and requires a specialised assembly of sequencing reads. Illumina miseq platform is a tool that has been used for whole genome sequencing as it provides high quality contig assemblies. These contigs can be assembled to a reference genome using programmes such as Velvet. (Hasman *et al.*, 2014).

Genome sequencing produces a vast array of data which can only be explored through the use of powerful computing and bioinformatics. Bioinformatics can be defined as the application of computerised systems to comprehend and assemble the information linked to biological macromolecules (Luscombe *et al.*, 2001). Visualising genome sequencing data is complicated by the vast amount of data produced , therefore there are standard methods that are applied to visualise sections of DNA, comparing sections of DNA and describing sections of DNA.A BLAST ring, nucleotide BLAST, and an amino acid pileup all produce visual outputs that can be used to interpret and analyse sequences by searching for primary differences in genome size as well as more in-depth analysis such as searching for candidate genes and single nucleotide polymorphisms (SNPs).

One of the most commonly applied bioinformatics tools is that of the BLAST search (Basic Local Alignment Search). This takes a query sequence and searches the genome sequence to look for the presence of particular genome sequences. Where those genome sequences exist within the genome being interrogated then the base matches are revealed by a vertical line between the two corresponding nucleotides and a gap between the two sequences is revealed represents a nucleotide base substitution.

Translations of nucleotides into amino acids can also be interrogated in a similar manner using a BLAST search of a translated genome. An amino acid pileup aligns multiple genomes and compares if the Single Nucleotide Polymorphisms (SNP's) in genes result in a change in amino acid sequence.

Finally the whole genome sequence can be visualised using a gross exploratory method such as a BLAST ring. Multiple genomes are displayed as coloured rings on a single diagram and it demonstrates the areas of similarity and differences between multiple whole genome sequences at a relatively crude yet comparative level. (Alikhan *et al.*, 2011).

Due to time constraint, it was impossible to search for all genes associated with *Salmonella* survival and virulence therefore a small gene pool was created which consisted of fur*, glgC, hilA, proP, ompR* and *rpoS. Salmonella* are capable of responding to changes in the extracellular environment and can regulate the expression of genes, a few of which rely on two-component systems. *OmpR* acts to changes in osmolarity and regulates invasion in addition to being involved in intracellular survival. In order to successfully establish infection, pathogens rely on the expression of virulence associated genes which are activated by a set of regulators. One global regulator is *hilA*, it works as a transcriptional regulator for SPI1, SPI4 and SPI5 which collectively mediate host cell invasion (Garai *et al.*, 2012). *Fur* (ferric uptake regulator) acts in response to iron and regulates genes that encode iron transport systems, virulence factors and metabolic enzymes (Ikeda *et al.*, 2005; Somerville and Proctor, 2009). Survival at suboptimal conditions is important for organisms in the food factory environment, evidence suggests that *Salmonella* can survive up to 100 weeks on plastic under desiccation. The *proP* gene plays a key role in the survival of *Salmonella* in low moisture foods and in mutants where the *proP* gene was deleted survival was lower and undetectable after 4 weeks (Finn *et al.*, 2013b). The alternative sigma factor *rpoS* also is key in *Salmonella* virulence; it mediates the expression of genes involved in resistance to environmental stresses including starvation, low pH and oxidation (Nickerson and Curtiss, 1997; Dodd and Aldsworth, 2002). The *glgC* gene has also been reported to play a part in survival of *Salmonella*, glycogen is widely available in enteric bacteria and it has been suggested that under environmental and nutritional stresses a store of energy storage compounds such as glycogen may play a vital role in survival at suboptimal temperatures (McMeechan *et al.*, 2005).

Therefore the aim of this chapter was to compare the genomes of *Salmonellae* from factory, clinical and veterinary environments to investigate any underlying changes at the genomic level which may account for the persistence of the factory strains. This was achieved by the specific objectives of:

- isolating and purifying the DNA from the isolates
- Submitting the extracted DNA to the Wellcome Trust Centre For Human Genetics (Oxford, UK), where the High-Throughput Sequencing facility uses an Illumina Miseq system to sequence strains.
- Assembling the contigs into genomic sequences and applying bioinformatics to visualise and explore the DNA to reveal similarities or differences

7.2 Materials and methods

Table 20 The panel of eight isolates of *Salmonella* selected for sequencing

Strain	Origin/environment	
*S.*Schwarzengrund	FSLS5-458 American clinical	*Salmonella* 1
*S.*Livingstone	factory	*Salmonella* 2
*S.*Senftenberg	factory	*Salmonella* 3
*S.*Senftenberg	clinical	*Salmonella* 4
*S.*Senftenberg	Veterinary	*Salmonella* 5
*S.*Kedougou	factory	*Salmonella* 6
*S.*Schwarzengrund	USA factory	*Salmonella* 7
*S.*Schwarzengrund	Veterinary	*Salmonella* 8

Table 20: the panel of eight isolates compiled for sequencing were labelled as *Salmonella* 1 to *Salmonella* 8.

The Wellcome laboratory provided DNA extraction guidelines, in which they stated a requirement of 200µg of DNA normalized to a concentration of 10ng/µl in 10mM Tris-Cl, pH 8.5. Following extraction the Nanodrop (Thermo,UK) was used to confirm that the 260/280 ratio was between 1.8 and 2 and that the 260/230 ratio was between 2-2.2. The material was then electrophoresed through a 0.7% agarose gel where samples of sufficient quality for sequencing gave distinct bands with little smearing.

7.2.1 DNA Extraction

DNA was extracted from eight *Salmonella* strains using the Thermo Scientific GeneJET Genomic DNA Purification Kit k0722 and the Gram-Negative Bacteria Genomic DNA Purification protocol, briefly summarised below with modifications. All reagents were provided in the kit unless stated otherwise.

Overnight cultures were grown in nutrient medium at 37°C in a shaking table top incubator (Fisher Scientific, UK) for 24 hours to achieve a final suspension of 2×10^9 bacterial cells per ml. A 1500µL volume of the overnight suspension was added to a 2 mL micro centrifuge tube (Star lab, UK) and centrifuged for 10 min at 5000 × g. The supernatant was discarded and this step was repeated with a further 1500µL of culture. Following discard, the tubes with the pellet were centrifuged again at 5000 × g for 60 seconds and any remaining supernatant was discarded.

The pellet was then re-suspended in 180 µL of ATL Digestion Solution and mixed by vortexing for 30 seconds. Next, 20 µL of Proteinase K Solution was added and mixed thoroughly by pipetting three times and vortexing for 30 seconds to obtain a uniform suspension. The tubes were then incubated at 56°C on a rocking platform for 30 minutes, while mixing by vortex every 10 minutes, until the cells were completely lysed. A 20 µL volume of RNase A Solution was then added and mixed by vortexing. The mixture was incubated for 10 minutes at ambient temperature. Following incubation, 200 µL of Lysis Solution was added to the sample and mixed thoroughly by vortexing for 15s until a homogeneous mixture was obtained. Next , 400 µL of 50% ethanol was added and mixed by vortexing, the prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube. The column was centrifuged for 1 min at 6000 × g. The collection tube containing the flow-through solution was discarded and the GeneJET Genomic DNA Purification Column was placed into a new 2 mL collection tube. A 500 µL of Wash Buffer I (with ethanol added) was added to the column and centrifuged for 1 min at 8000 × g. The flow-through was discarded and the purification column was placed back into the collection tube. Next, 500 µL of Wash Buffer II (with ethanol added) was added to the GeneJET Genomic DNAPurification Column and centrifuged for 3 min at maximum speed (≥12000 × g). To remove any residual solution in the purification column, the collection tube was emptied and the column was re-spun for one min at maximum speed, the collection tube containing the flow-through solution was discarded and the GeneJET Genomic DNA Purification Column was transferred into a sterile 1.5 mL

microcentrifuge tube. Following this, 200 µL of Elution Buffer was added to the centre of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA and incubated for 2 min at room temperature and centrifuged for 1 min at 8000 × g. This step was repeated with an additional 200 µL of Elution Buffer for maximum yield. Lastly, the purification column was discarded and the purified DNA was quantified using the Nanodrop (Thermo, UK), the 260/280 ratio and the 260/230 ratios were noted and the purified DNA stored at -20°C.

7.2.2 *DNA Concentration & purification*

To improve purity and further concentrate the extracted DNA, it was purified using the QIAquick PCR purification kit in accordance with the manufacturer's instructions, with the exception that DNA was eluted from the column using 10mM Tris-Cl, pH 8.5.

7.2.3 *Agarose gel electrophoresis*

Agarose gels were prepared using 1x TAE buffer (0.04M Tris- acetetate, 0.001 M EDTA pH 8.5) and Molecular Biology Grade Agarose (Geneflow, Staffordshire). A Duran bottle was weighed and 1.4g agarose was added into 200ml of 1x TAE buffer and stirred for one minute. The total weight was measured and the solution was heated using a microwave for one minute with the lid removed. Following heating it was removed from the microwave, stirred for an additional minute and then returned to the microwave and heated until the solution was dissolved and clear. The bottle was reweighed and any loss due to evaporation was replaced with fresh1x TAE buffer. The solution was allowed to cool to approximately 55°C with constant stirring. Ethidium bromide was added to a final concentration of 0.5µg/ml. Gels were poured in a sealed gel tray containing a single 20 welled comb. Gels were cast at 5-8mm in dimensions and allowed to set at ambient temperature for 30 minutes. The gel was then transferred to the electrophoresis unit and submerged in 1xTAE buffer and the comb was removed. Approximately 500ng of DNA was loaded into each well using a loading buffer. Gels were electrophoresed at 5 volts per cm potential difference, until the dye front had migrated 2/3 down the gel. Gels were visualised using G Box HR 16 under UV trans illumination with data acquired using the GENE Sys software (Syngene,UK).

Next, the Purified DNA was submitted to the Wellcome Trust Centre for Human Genetics (Oxford, UK). A 20 µl volume of DNA was transported on dry ice in labelled micro centrifuge tubes, where the High-Throughput Sequencing facility used an Illumina Miseq system to sequence strains. The genome sequences were recovered from a portal with links to the assembled genome sequences.

7.2.4 Bioinformatics

The data were analysed in collaboration with a bioinformatician at Aston University. Firstly a BLAST ring was generated, and this is a visual representation which shows similarities between a reference sequence and other sequences as concentric rings. The rings were produced using the online tool BLAST Ring Image Generator (BRIG) following the online installation instructions and manual (Alikhan *et al.*, 2011).

Next, the presence or absence of candidate genes was noted using a Nucleotide BLAST (Basic Local Alignment Search Tool). These data were generated using the tool available BLAST from the ftp site at NCBI, installing it on a PC and writing some `perl wrappers' that automated the analysis of multiple sequences and BLASTed against the 8 different genomes. Lastly, a translated BLAST was undertaken on the amino acids, this aligns multiple genomes and compares if the SNPs in the genes resulted in a change in the amino acid sequence. The sequences were translated with a `Perl programme' and aligned using an online tool called ClustalX.

7.3 Results

The table below demonstrates the purity and concentration of the DNA submitted for sequencing.

Table 21 Concentration and purity of the extracted DNA using Nanodrop

isolate number	Strain	Concentration (ng/ul)	Volume (ul)	OD 260/280	OD 260/230
isolate 1	*S.*Schwarzengrund clinical	87.00	20.00	1.84	2.08
isolate 2	*S.*Livingstone factory	45.00	20.00	1.83	2.00
isolate 3	*S.*Senftenberg factory	84.00	20.00	1.88	2.16
isolate 4	*S.*Seftenberg clinical	75.20	20.00	1.83	2.00
isolate 5	*S.*Senftenberg vet	42.50	20.00	1.80	2.07
isolate 6	*S.*Kedougou factory	53.80	20.00	1.83	2.01
isolate 7	*S.*Schwarzengrund factory	81.00	20.00	1.88	2.16
isolate 8	*S.*Schwarzengrund vet	69.60	20.00	1.84	2.14

Table 21 shows the concentration of the DNA that was extracted for each isolate and values indicating the purity of the DNA. The ratio of absorbance at 260 nm and 280 nm is used as an assessment of the purity of DNA, generally a ratio of around 1.8 is considered as pure for DNA and all the values above are at this value or above. The 260/230 ratio is used as a secondary measure of nucleic acid purity. Often the value obtained for "pure" nucleic acid is higher than the 260/280 values. The normal range for this ratio is between 2.0-2.2 and again all the ratios for the extracted DNA are within this range. If either of the two ratios were lower than the expected values it would indicate the presence of contamination. The concentration of DNA extracted varied across the eight isolates, with values ranging from 45.00 to 87.00 ng/ul, for sequencing 10ng/µl was required, therefore a 20µl volume was dispensed into an Eppendorf tube and submitted for sequencing. The concentrations in Table 21 were sent to allow more accurate quantification at the Wellcome centre using their Qbit fluorimeter. It must be noted that the nanodrop overestimates the amount of material present and sole reliance on this method of quantification may risk the library failing.

Figure 87 Agarose gel image from the extracted genomic DNA

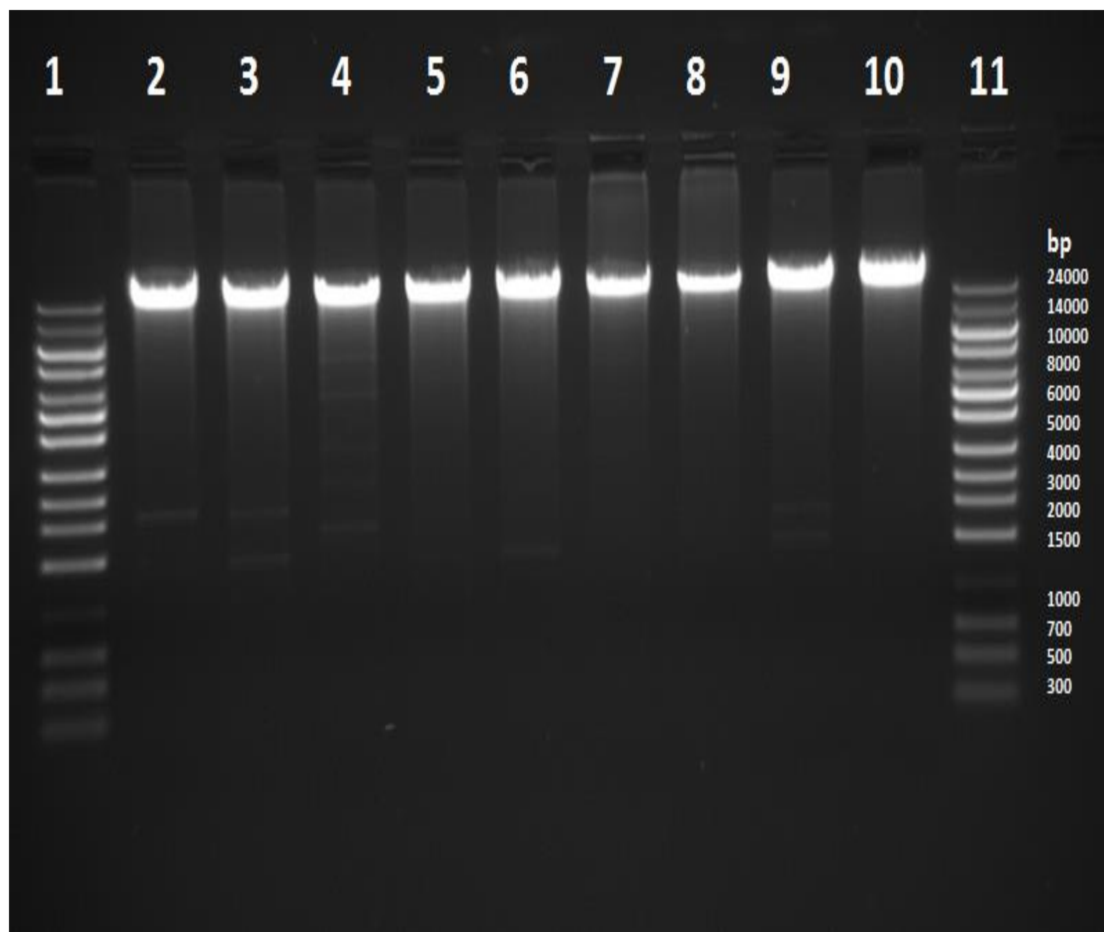


Figure 87: 0.7% Agarose gel image from the extracted genomic DNA. (1) UltraRanger 1kb DNA Ladder (Norgen Biotek Corp) (2) *S.*Schwarzengrund factory (3) *S.*Schwarzengrund vet (4) *S.*Schwarzengrund clinical (5) *S.*Senftenberg 775W (6) *S.*Senftenberg factory (7) *S.*Senftenberg veterinary (8) *S.*Livingstone factory (9) *S.*Kedougou factory (10) *S.*Montevideo factory (11) UltraRanger 1kb DNA Ladder. Genomic DNA Samples 2-9 can be visualised by the clear bands with minimal smearing suggesting the DNA was intact.

7.3.1 *De novo assembly with Velvet*

All the reads for each sample were assembled with Velvet. Assemblies were generated for each odd k-mer between 75 and 149 by Velvet version 1.2.10 initiated by Velvet Optimiser version 2.2.5.

Table 22 The size and base content of the eight sequenced genomes

Strain	Contigs	Longest contig	Total bases	Base content
*S.*Schwarzengrund FSL S5-458 American clinical	86	1,282,017	4,776,171	A: 1139225 %: 0.234 G: 1250136 %: 0.257 C: 1238369 %: 0.255 T: 1144334 %: 0.235
*S.*Livingstone Factory	68	1,735,613	4,836,059	A: 1154091 %: 0.235 G: 1271215 %: 0.258 C: 1246119 %: 0.253 T: 1157613 %: 0.235
*S.*Senftenberg Factory	88	787,101	4,840,647	A: 1155441 %: 0.235 G: 1262908 %: 0.257 C: 1252223 %: 0.254 T: 1162555 %: 0.236
*S.*Senftenberg (775W) Clinical	166	1,438,806	5,242,563	A: 1256044 %: 0.236 G: 1366912 %: 0.256 C: 1347956 %: 0.253 T: 1261396 %: 0.237
*S.*Senftenberg Veterinary	86	1,684,902	4,956,196	A: 1178759 %: 0.234 G: 1323247 %: 0.263 C: 1250651 %: 0.248 T: 1196028 %: 0.237
*S.*Kedougou Factory	80	1,014,823	4,815,920	A: 1157987 %: 0.236 G: 1249832 %: 0.255 C: 1249824 %: 0.255 T: 1155045 %: 0.236
*S.*Schwarzengrund Factory	62	746,978	4,620,244	A: 1107191 %: 0.235 G: 1195167 %: 0.254 C: 1210933 %: 0.258 T: 1101757 %: 0.234
*S.*Schwarzengrund Veterinary	62	1,367,780	4,570,578	A: 1091139 %: 0.235 G: 1199268 %: 0.258 C: 1182007 %: 0.254 T: 1093505 %: 0.235

Table 22: illustrates the size of the eight sequenced genomes. Generally the size of *Salmonella* is reported as ~4.7 megabases. The genome size of the factory isolates showed variation and ranged between 4.69 to 4.91mb. The smallest genome size within the factory isolates was reported for *S.*Schwarzengrund and was approximately 200,000 bases smaller (2%) in comparison to other factory isolates. The veterinary isolate of *S.*Schwarzengrund was also small in comparison to other isolates (4.64mb). However it is unlikely 100% coverage was obtained for these 2 genomes. The largest genome was illustrated for *S.*Senftenberg 775W (5.32mb) and the veterinary isolate of *S.*Senftenberg (5.03mb). This indicates that some of the isolates of *Salmonella* are likely to have acquired genetic information through horizontal gene transfer.

7.3.2 Basic alignment search tool (BLAST)

Any gross genomic differences were explored and visualised using BLAST. A Blast ring image generator (BRIG) is an online tool that was used to produce rings that graphically display circular comparisons across multiple bacterial genomes. An initial diagram was produced with concentric rings representing each of the eight isolates around the selected reference genome (*S.*Typhimurium SL1344), followed by rings to show just the serotype matched isolates and the factory isolates in comparison to each other.

Figure 88 Summary BRIG output image displaying eight *Salmonella* isolates

Figure 88: BRIG output shows reference genome *S*.Typhimurium SL1344 compared against eight sequenced *Salmonella* genomes. All the isolates display some degrees of similarity, for example various regions of the reference strain are missing in the eight isolates (around 1000kbp, 2400kbp & 2800kbp) as shown by breaks coloured white in the rings. The breaks in the ring also show, that in terms of gene content the factory, veterinary and clinical isolates of *S*.Schwarzengrund are very similar if not almost identical. Although, no major genomic differences across the isolates can be visualised, at this level any differences can only be speculated as areas of the genome would require further analysis via PCR for more in depth understanding.

Figure 89 BRIG output image displaying the factory *Salmonella* isolates

Figure 89: BRIG output shows the four sequenced factory *Salmonella* genomes. There are notable areas of similarity displayed across various regions of the rings. Although there are some distinctive breaks present some of the isolates (e.g *S*.Livingstone at ~ 750kb and ~4250 kbp) at this level it is not possible to explain the results further however differences may be linked to serotype rather than environment.

Figure 90 BRIG output image displaying the serotype matched isolates for *S*.Senftenberg

Figure 90: BRIG output shows the three serotype matched isolates of the factory, clinical and veterinary for *S*.Senftenberg. The output displays notable areas of similarity across the regions of the three rings as shown by the distinctive pattern.

Figure 91 BRIG output image displaying the serotype matched isolates for *S.*Schwarzengrund

Figure 91: BRIG output shows the three serotype matched isolates of the factory, clinical and veterinary for *S*.Schwarzengrund. The output displays notable areas of similarity across the regions of the three rings as shown by the distinctive pattern.

The BRIG outputs have provided an overall insight into the genomic sequences. To further explore these data, a panel of genes was selected that were known to be involved in environmental survival and persistence of *Salmonella.* The presence or absence of these genes in each of the eight isolates was visualised using a Nucleotide BLAST, any SNP's in the query gene in comparison to the reference were noted and explored further in an amino acid pileup.

7.3.3 *Nucleotide BLAST Results*

All six of the genes searched for were present in the eight isolates of *Salmonella.* Below are examples of the nucleotide sequences for a regulatory gene (*fur*) and a structural gene (*ompR*). Homology between the reference and the query sequence is revealed with a vertical line between the 2 nucleotide bases; if the nucleotide bases are different in the query, this is shown by a gap between the two sequences.

Figure 92 Nucleotide sequence for regulatory gene *fur*

```
Query  1      ATGACTGACAACAATACCGCATTAAAGAAGGCTGGCCTGAAAGTAACGCTTCCTCGTTTA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62535  ATGACTGACAACAATACCGCATTAAAGAAGGCTGGCCTGAAAGTAACGCTTCCTCGTTTA  62594

Query  61     AAAATTCTGGAAGTTCTTCAGGAACCAGATAACCATCACGTCAGTGCGGAAGATTTATAC  120
              |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  62595  AAAATTCTGGAAGTTCTTCAGGAGCCAGATAACCATCACGTCAGTGCGGAAGATTTATAC  62654

Query  121    AAACGCCTGATCGACATGGGTGAAGAAATCGGTCTGGCAACCGTATACCGTGTGCTGAAC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62655  AAACGCCTGATCGACATGGGTGAAGAAATCGGTCTGGCAACCGTATACCGTGTGCTGAAC  62714

Query  181    CAGTTTGACGATGCCGGTATCGTGACCCGCCATAATTTTGAAGGCGGTAAATCCGTTTTT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62715  CAGTTTGACGATGCCGGTATCGTGACCCGCCATAATTTTGAAGGCGGTAAATCCGTTTTT  62774

Query  241    GAACTGACGCAACAGCATCATCACGACCATCTTATCTGCCTTGATTGCGGAAAAGTGATT  300
              |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  62775  GAACTGACGCAACAGCATCATCACGACCATCTTATCTGCCTTGACTGCGGAAAAGTGATT  62834

Query  301    GAATTTAGTGATGACTCTATTGAAGCGCGCCAGCGTGAAATTGCGGCGAAACACGGTATT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62835  GAATTTAGTGATGACTCTATTGAAGCGCGCCAGCGTGAAATTGCGGCGAAACACGGTATT  62894

Query  361    CGTTTAACTAATCACAGCCTCTATCTTTACGGCCACTGCGCTGAAGGCGACTGCCGCGAA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62895  CGTTTAACTAATCACAGCCTCTATCTTTACGGCCACTGCGCTGAAGGCGACTGCCGCGAA  62954

Query  421    GACGAGCACGCGCACGATGACGCGACTAAATAA  453
              |||||||||||||||||||||||||||||||||
Sbjct  62955  GACGAGCACGCGCACGATGACGCGACTAAATAA  62987
```

Figure 92 shows an example of the nucleotide sequence of the shortest gene (*fur*) in the clinical isolate of *S*.Schwarzengrund. Over the length of the 453 nucleotides of the *fur* gene, 451 bases are identical, this is shown by the presence of the vertical lines and represents 99% identity between the query and reference strain. There are no gaps over the length of the gene; however there are two single nucleotide polymorphisms (SNP) one at positions 85 whereby Adenine in the query is replaced with Guanine and also at position 285 whereby Thymine in the query is replaced with Cytosine. These were explored later in the translated BLAST.

Figure 93 Nucleotide sequence of structural gene *ompR*

```
Query  1      ATGCAAGAGAATTATAAGATTCTGGTGGTTGATGACGATATGCGTCTGCGGGCGCTACTG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87553  ATGCAAGAGAATTATAAGATTCTGGTGGTTGATGACGATATGCGTCTGCGGGCGCTACTG  87494

Query  61     GAACGTTATCTGACCGAGCAGGGCTTCCAGGTTCGAAGCGTCGCTAACGCTGAGCAGATG  120
              |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  87493  GAACGTTATCTGACCGAGCAGGGCTTCCAGGTTCGAAGCGTCGCTAACGCCGAGCAGATG  87434

Query  121    GATCGTCTGCTGACCCGTGAATCTTTCCATCTCATGGTACTGGATTTAATGCTGCCAGGT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87433  GATCGTCTGCTGACCCGTGAATCTTTCCATCTCATGGTACTGGATTTAATGCTGCCAGGT  87374

Query  181    GAAGATGGTCTGTCGATTTGTCGTCGCCTGCGTAGTCAAAGTAATCCAATGCCGATCATT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87373  GAAGATGGTCTGTCGATTTGTCGTCGCCTGCGTAGTCAAAGTAATCCAATGCCGATCATT  87314

Query  241    ATGGTCACGGCGAAGGGTGAAGAGGTTGACCGTATCGTCGGGCTGGAAATCGGCGCCGAT  300
              |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  87313  ATGGTCACGGCGAAGGGTGAAGAAGTTGACCGTATCGTCGGGCTGGAAATCGGCGCCGAT  87254

Query  301    GACTACATTCCTAAACCGTTTAACCCGCGCGAGCTGTTGGCGCGTATTCGCGCCGTGTTA  360
              ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  87253  GACTACATTCCTAAACCGTTTAACCCGCGCGAGCTGTTGGCGCGTATCCGCGCCGTGTTA  87194

Query  361    CGTCGTCAGGCAAACGAACTGCCCGGCGCGCCGTCGCAGGAAGAGGCCGTTATCGCGTTC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  87193  CGTCGTCAGGCAAACGAACTGCCCGGCGCGCCGTCGCAGGAAGAGGCCGTTATTGCGTTC  87134

Query  421    GGTAAGTTTAAACTGAACCTCGGTACGCGCGAGATGTTCCGTGAAGATGAACCGATGCCG  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87133  GGTAAGTTTAAACTGAACCTCGGTACGCGCGAGATGTTCCGTGAAGATGAACCGATGCCG  87074

Query  481    CTGACCAGCGGGGAGTTTGCGGTACTGAAAGCGTTAGTCAGCCATCCGCGCGAGCCGCTC  540
              ||||||||||||||||||||||||||||||||| | || ||||| |||||||||||||||
Sbjct  87073  CTGACCAGCGGGGAGTTTGCGGTACTGAAAGCGCTGGTAAGCCACCCGCGCGAGCCGCTC  87014

Query  541    TCTCGCGATAAGCTGATGAATCTGGCCCGTGGCCGCGAGTATTCCGCGATGGAACGCTCC  600
              |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87013  TCTCGCGACAAGCTGATGAATCTGGCCCGTGGCCGCGAGTATTCCGCGATGGAACGCTCC  86954

Query  601    ATCGACGTCCAGATCTCCCGCCTGCGCCGTATGGTGGAAGAAGATCCGGCACATCCGCGT  660
              |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  86953  ATCGACGTCCAGATCTCCCGCCTGCGCCGTATGGTGGAAGAAGATCCGGCGCATCCGCGT  86894

Query  661    TATATTCAGACCGTCTGGGGCCTGGGCTACGTCTTTGTACCGGACGGTTCTAAAGCATGA  720
              ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  86893  TATATTCAGACCGTCTGGGGCCTGGGCTACGTCTTCGTACCGGACGGTTCTAAAGCATGA  86834
```

Figure 93: shows the *ompR* in the clinical isolate of *S.*Schwarzengrund. Over the length of the 720 nucleotides of the *ompR* gene, 709 bases are identical, representing 98% identity between the query (*ompr*) and reference. There were no gaps over the length of the gene; single nucleotide polymorphisms (SNP) could be observed across the gene. Firstly at position 111 whereby Thymine in the query is replaced with cytosine, secondly at position 263, Guanine is replaced with Adenine. At position 348 Thymine is replaced with Cytosine. At position 414 Cytosine was replaced with Thymine. At position 514 Thymine is replaced with Cytosine. At position 516 Adenine is replaced with Guanine. At position 519 Cytosine is replaced with Adenine. At positions 525 and 549 and 696, Thymine is replaced with Cytosine and at position 651 Adenine is replaced with Guanine. These were explored later in the translated BLAST. The remainder of the SNP's were recorded in tables for each of the six genes followed by the outputs from the amino acid pileup.

Table 23 Lists the SNP's observed in the *fur* gene across the eight isolates of *Salmonella*

Fur gene						
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	No.of SNP's	Position of substitution	Substitution
1) *S.*Schwarzengrund clinical	0	453	451	2	85 285	A > G T > C
2) *S.*Livingstone factory	0	453	452	1	285	T > C
3) *S.*Senftenberg factory	0	453	451	1	285	T > C
4) *S.*Senftenberg 775W	0	453	451	1	285	T > C
5) *S.*Senftenberg Veterinary	0	453	452	1	285	T > C
6) *S.*Kedougou Factory	0	453	452	1	285	T > C
7) *S.*Schwarzengrund factory	0	453	451	2	85 285	A > G T > C
8) *S.*Schwarzengrund veterinary	0	453	451	2	85 285	A > G T > C

Table 23: the *fur* gene contains 453 nucleotides and there were no gaps in any of the isolates. All the isolates contained one SNP at position 285 whereby Thymine was substituted with Cytosine; however the factory, clinical and veterinary isolates of *S.*Schwarzengrund included an additional SNP at position 85 whereby Adenine was replaced with Guanine. These SNP's were explored later in the translated BLAST to identify if they resulted in an amino acid change.

Figure 94 Translated amino acid pileup of the *fur* gene sequence across eight isolates of *Salmonella*

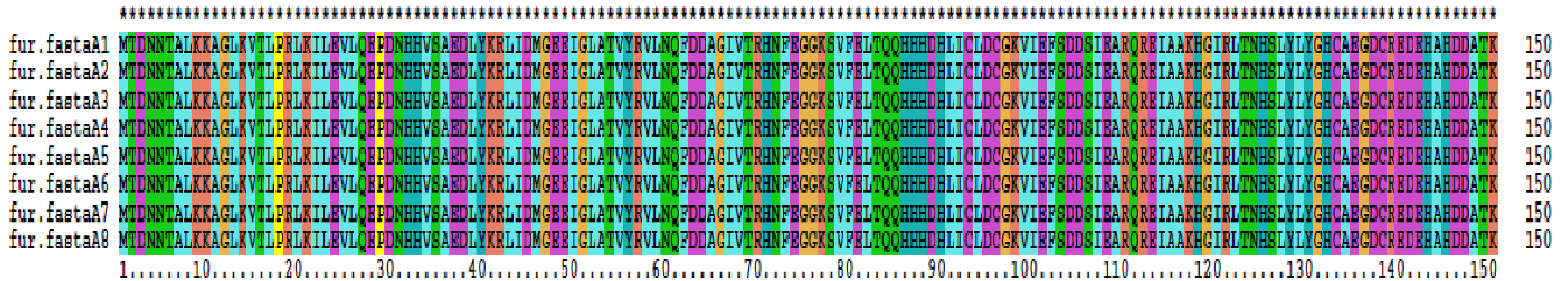


Figure 94: represents a translated pileup of the *fur* gene. There are 150 amino acids and the output shows that this gene is conserved across all eight isolates of *Salmonella* and no point mutations or substitutions can be visualised.

Table 24 Lists the SNP's observed in the *glgC* gene across the eight isolates of *Salmonella*

GlgC gene						
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	No. of SNP's	Position of substitution	Substitution
1) *S.*Schwarzengrund clinical	0	1296	1292	4	52 483 585 676	T > C G > A A > G A > G
2) *S.*Livingstone factory	0	1296	1291	5	625 705 855 981 1121	C > G T > C G > A T > C G > A
3) *S.*Senftenberg factory	0	1296	1294	2	625 969	T > G T > A
4) *S.*Senftenberg 775W	0	1296	1293	3	243 625 705	G > A T > G T > G
5) *S.*Senftenberg Veterinary	0	1296	1294	2	625 969	T > G C > A
6) *S.*Kedougou Factory	0	1296	1292	4	312 462 625 1011	C > T C > T T > G C > T
7)*S.*Schwarzengrund factory	0	1296	1292	4	52 483 585 675	T > C G > A A > G A > G
8) *S.*Schwarzengrund veterinary	0	1296	1292	4	52 483 585 675	T > C G > A A > G A > G

Table 24: The *glgC* gene contains 1296 m nucleotides and there were no gaps in any of the isolates. The number of SNP's varied with isolate. These SNP's were explored in the translated BLAST to identify if they resulted in an amino acid change.

Figure 95 Translated amino acid pileup of the glgC gene sequence across eight isolates of *Salmonella*

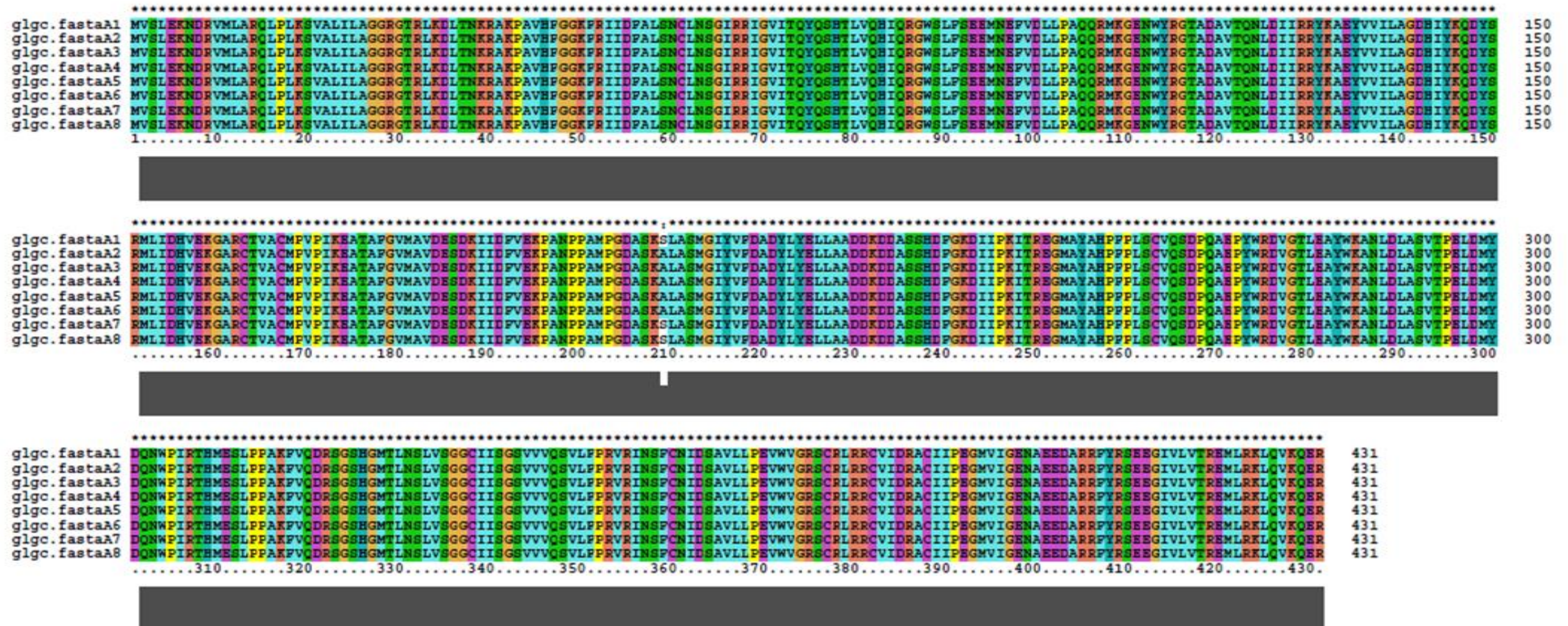


Figure 95: represents a translated amino acid pileup of the *glgC* gene (Glucose-1-phosphate adenylyltransferase) sequence, showing changes at around position 209/ 210 in *Salmonellas* 1, (*S.*Schwarzengrund FSL S5-458 American clinical), 7 (*S.*Schwarzengrund factory) and 8 (*S.*Schwarzengrund veterinary). This seems to be a change linked to serotype whereby the amino acid A (Alanine) is replaced by S (Serine) representing a single nucleotide polymorphism (often referred to as a SNP) which causes a substitution in amino acids.

Table 25 Lists the SNP's observed in the *ompR* gene across the eight isolates of *Salmonella.*

Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	Number of SNP's	Position and substitution
1) *S.*Schwarzengrund clinical	0	720	709	11	111 T > C 519 C > A 264 G > A 525 T > C 348 T > C 549 T > C 414 C > T 696 T > C 514 T > C 651 A > G 516 A > G
2) *S.*Livingstone factory	0	720	712	8	111 T > C 549 T > C 150 T > C 573 C > T 219 A > G 669 G > A 264 G > A 679 C > A
3) *S.*Senftenberg factory	0	720	715	5	111 T > C 358 T > C 264 G > A 674 G > A 354 C > T
4) *S.*Senftenberg 775W	0	720	716	4	111 T > C 358 T > C 348 T > C 675 C > A
5) *S.*Senftenberg Veterinary	0	720	715	5	111 T > C 264 G > A 150 T > C 675 C > A 219 A > G
6) *S.*Kedougou Factory	0	720	715	5	111 T > C 651 A > G 348 T > C 696 T > C 358 T > C
7) *S.*Schwarzengrund factory	0	720	709	11	111 T > C 519 C > A 264 G > A 525 T > C 348 T > C 549 T > C 414 C > T 651 A > G 514 T > C 696 T > C 516 A > G
8) *S.*Schwarzengrund veterinary	0	720	709	11	111 T > C 519 C > A 264 G > A 525 T > C 348 T > C 549 T > C 414 C > T 651 A > G 514 T > C 696 T > C 516 A > G

Table 25: The *ompR* gene contains 720 nucleotides and there were no gaps in any of the isolates. There were multiple SNP's across the gene. The highest numbers of SNP's were observed for the three *S.*Schwarzengrund isolates all at identical positions. These SNP's were explored in the translated BLAST to identify if they resulted in an amino acid change

Figure 96 Translated amino acid pileup of the *ompR* gene sequence across eight isolates of *Salmonella*

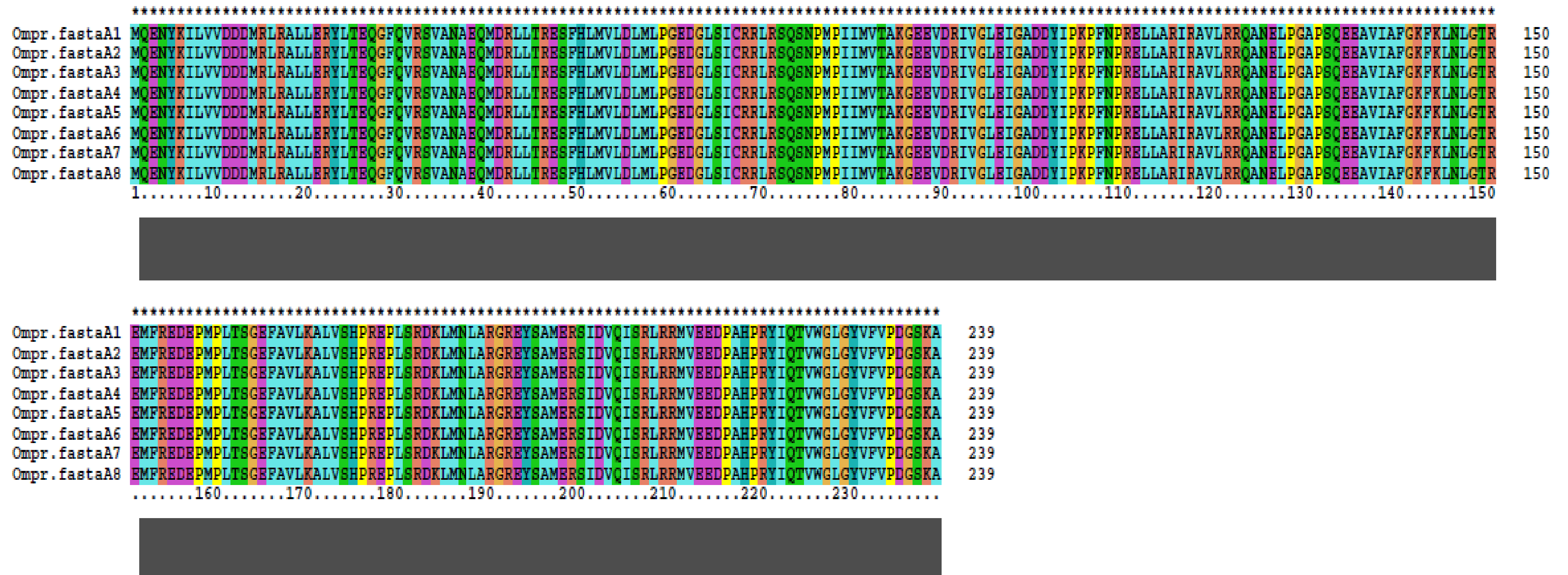


Figure 96: represents a translated pileup of the *OmpR (*outer membrane porin gene). This gene is conserved across all isolates with no point mutations or substitutions that can be visualised. It is not unusual for outer membrane proteins or flagella to be different across isolates as there is selective pressure on organisms to modify appearance to suit the environment.

Table 26 Lists the SNP's observed in the *rpoS* gene across the eight isolates of *Salmonella*

Rpos gene						
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	No. of base pairs in query identical to subject	No. of SNP's	Position and substitution	
1) *S.*Schwarzengrund clinical	0	992	989	3	474 C > T 711 C > T	875 C > T
2) *S.*Livingstone factory	0	992	991	1	372 C > T	
3) *S.*Senftenberg factory	0	992	990	2	372 T > C	474 C> T
4) *S.*Senftenberg 775W	0	992	991	1	474 C > T	
5) *S.*Senftenberg Veterinary	0	992	990	2	372 C > T	474 C > T
6) *S.*Kedougou Factory	0	992	991	1	474 C > T	
7) *S.*Schwarzengrund factory	0	992	989	3	474 C > T 711 C > T	875 C > T
8) *S.*Schwarzengrund veterinary	0	992	989	3	474 C > T 711 C > T	875 C > T

Table 26: Lists the SNP's observed in *rpoS* across the eight isolates of *Salmonella.* The *rpoS gene* contains 992 nucleotides and there were no gaps in any of the isolates. There were not many SNP's in the gene and the same pattern was observed whereby all three isolates of *S.*Schwarzengrund had the same number of SNP's at the same position. These SNP's were explored in the translated amino acid BLAST to identify if the substitutions resulted in an amino acid change.

Figure 97 translated amino acid pileup of the *rpoS* sequence across eight isolates of *Salmonella*

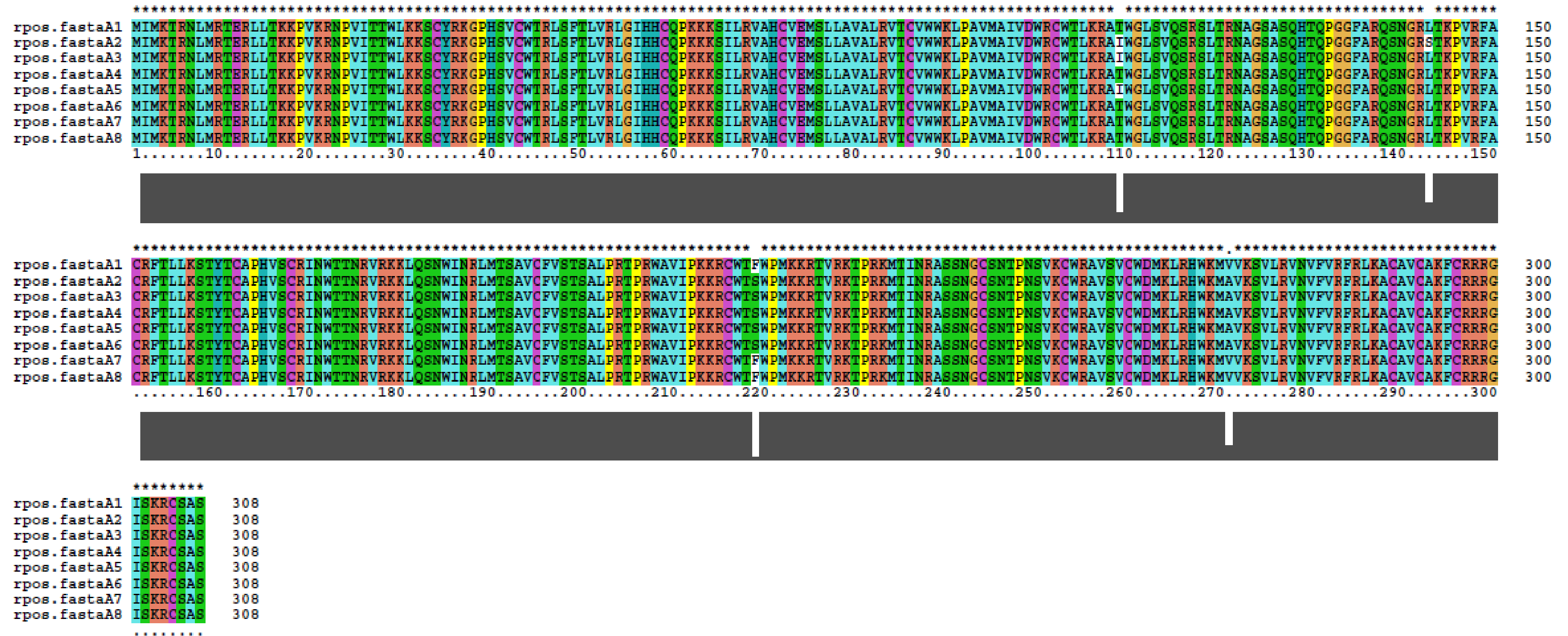


Figure 97: represents a translated pileup of the 308 amino acids in the *rpoS* (RNA polymerase, sigma S) sequence, which is an alternative sigma factor involved in the stationary phase. It shows amino acid substitutions in four positions. Firstly at around position 110 in 2 (*S*.Livingstone factory), 3 (*S*.Senftenberg factory) and 5 (*S*.Senftenberg veterinary) whereby T (Threonine) is substituted with I (Isoleucine). At around position 143 in (*S*.Livingstone factory), L (Leucine) is replaced with S (Serine). In the clinical, factory and veterinary isolates of S.Schwarzengrund, at position 220, S (Serine) is replaced with F (Phenylalanine) in addition to position 272, whereby A (Alanine) is substituted with V (Valine).

Table 27 Lists the SNP's observed in the *hilA* gene across the eight isolates of *Salmonella*

hilA gene					
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	Position and substitution	
1) *S*.Schwarzengrund clinical	0	1662	1646	477 G > A 501 C > T 513 G > A 564 G > A 583 T > C 603 T > C 699 G > T 726 A > G	748 A > G 815 C > T 957 T > C 1052 C > T 1111 T > C 1146 T > C 1206 C > T 1355 G > T
2) *S*.Livingstone factory	0	1662	1646	165 G > C 520 C > T 575 C > T 603 T > C 726 A > G 816 C > T 957 T > C 1053 C > T	1146 T > C 1446 C > T 1494 A > G 1455 T > C 1467 T > C 1482 G > A 1506 T > A 1518 A > T
3) *S*.Senftenberg factory	0	1662	1645	504 C > T 522 T > C 582 T > C 603 T > C 726 A > G 957 T > C 1053 C > T 1146 T > C	1206 C > T 1308 C > T 1309 C > T 1351 C > A 1422 A > G 1446 C > T 1455 T > C 1494 A > G 1518 A > T
4) *S*.Senftenberg 775W	0	1662	1645	126 C > T 520 T > A 582 T > C 603 T > C 774 T > C 864 T > C 1053 C > T 1146 T > C	1446 C > T 1455 T > C 1467 T > C 1482 G > A 1494 A > A 1506 T > C 1518 T > G
5) *S*.Senftenberg eterinary	0	1662	1645	504 C > T 522 T > C 582 T > C 603 T > C 726 A > G 1053 C > T 1146 T > C 1206 C > T	1308 C > T 1309 C > T 1355 C > A 1422 A > G 1446 C > T 1455 T > C 1494 A > G 1506 T > C 1518 T > A

***HilA gene* (ii)**					
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	Position and substitution	
6) *S.*Kedougou Factory	0	1662	1645	292 C > T 503 C > T 522 T > C 592 T > C 603 T > C 954 C > T 957 C > T 1053 C > T	1110 T > C 1308 C > T 1309 C > T 1422 A > G 1446 C > T 1494 A > G 1506 T > C 1518 A > T
7)*S.*Schwarzengrun d factory	0	1662	1646	477 G > A 501 C > T 513 G > A 564 G > A 582 T > C 603 T > C 699 G > T 726 A > G 748 A > G	816 C > T 957 T > C 1010 T > C 1053 C > T 1110 T > C 1146 T > C 1206 C > T 1356 G > T
8)*S.*Schwarzengrun d veterinary	0	1662	1646	477 G > A 501 C > T 513 G > A 564 G > A 582 T > C 603 T > C 699 G > T 726 A > G 748 A > G	816 C > T 957 T > C 1053 C > T 1110 T > C 1146 T > C 1206 C > T 1356 G > T

Table 27 The *hilA gene* contains 1662 nucleotides and there were no gaps in any of the isolates. Multiple SNP's were seen across the gene in the eight isolates with identical SNP's visualised in the three *S.*Schwarzengrund isolates. These SNP's were explored in the translated amino acid BLAST to identify if the substitutions resulted in an amino acid change.

Figure 98 translated amino acid pileup of the *hilA* sequence across eight isolates of *Salmonella*

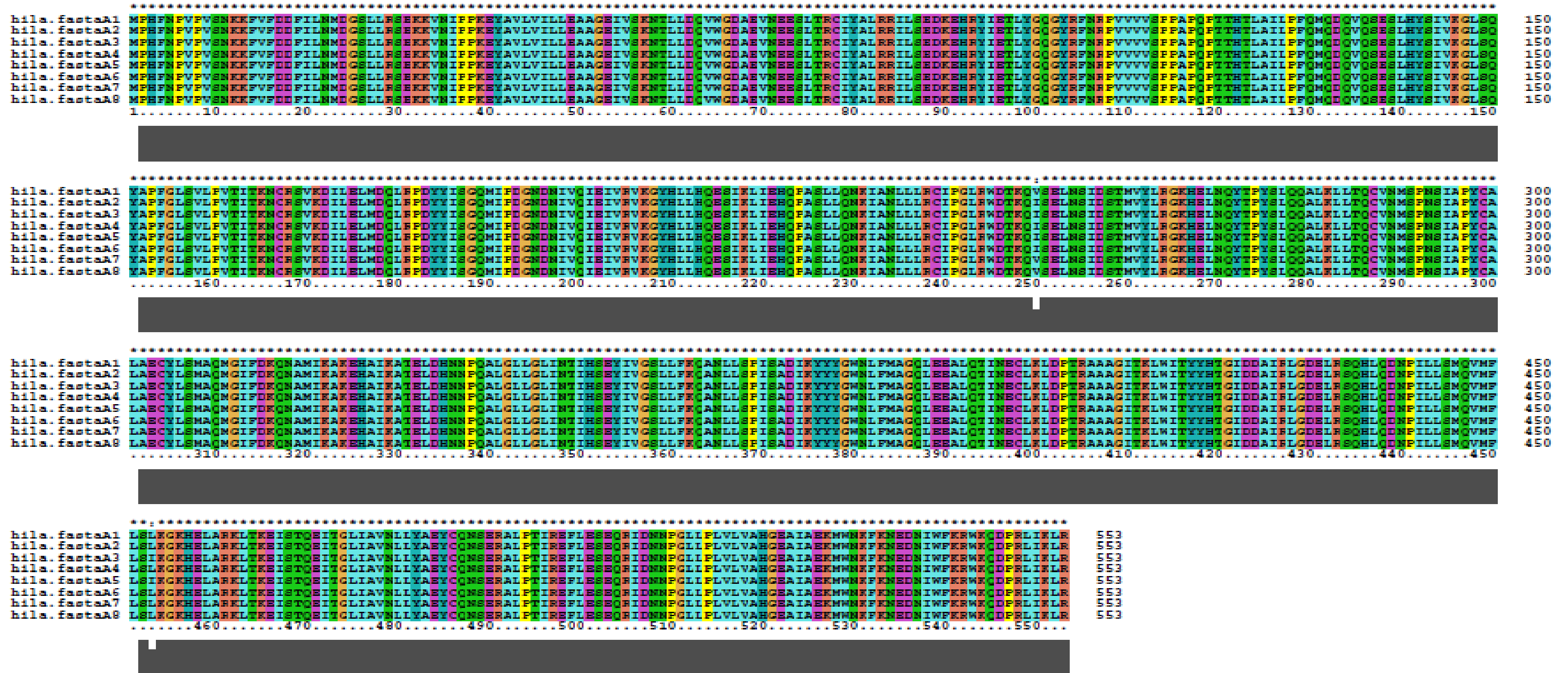


Figure 98: represents a translated pileup of the 553 amino acids in the *hilA* gene. Despite the multiple SNP's in the nucleotide sequence only two SNP in the amino acid sequence can be observed. Firstly at around position 250 in *Salmonellas* 1, (*S.*Schwarzengrund FSL S5-458 American clinical) 7 (*S.*Schwarzengrund factory) and 8 (*S.*Schwarzengrund veterinary) This seems to be a serotype effect whereby the amino acid I (Isoleucine) is substituted with V (Valine). Furthermore at position 453, in 2 (*S.*Livingstone factory) and 5 (*S.*Senftenberg veterinary), the amino acid L (Leucine) is replaced with I (Isoleucine). '

Table 28 Lists the SNP's observed in the *proP* gene across the eight isolates of *Salmonella*

prop gene (i)					
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	Position and substitution	
1) *S*.Schwarzengrund clinical	0	1503	1482	60 T > C 303 C > A 348 C > T 396 G > A 405 G > A 502 C > T 516 T > C 525 A > G 555 A > G 557 A > G	588 T > G 621 C > T 633 G > A 777 T > G 1068 A > C 1158 T > C 1297 A > G 1326 T > C 1443 T > C 1461 A > G 1491 T > C
2) *S*.Livingstone factory	0	1503	1491	69 G > A 416 T > C 525 A > G 555 G > A 561 G > A 666 G > A	972 A > T 978 A > G 1068 A > C 1158 T > C 1336 T > C 1368 C > T
3) *S*.Senftenberg factory	0	1503	1486	78 G > A 378 G > A 517 T > C 525 A > G 555 G > A 588 T > G 621 C > T 633 G > A	666 G > A 724 T > C 777 T > G 1068 A > C 1158 T > C 1204 C > T 1443 T > C 1491 T > C
4) *S*.Senftenberg 775W	0	1503	1484	516 T > C 525 A > G 555 G > A 561 G > A 573 C > A 579 G > T 588 T > G 621 C > T 633 G > A 825 C > T	834 G > A 978 A > G 1008 C > T 1068 A > C 1158 T > C 1296 A > G 1336 T > C 1392 C > T 1491 T > C

Prop gene (ii)					
Salmonella isolate	Gaps in sequence	Nucleotide sequence length	Number of base pairs in query identical to subject	Position and substitution	
5) *S*.Senftenberg Veterinary	0	1503	1486	78 G > A 378 G > A 516 T > C 525 A > G 555 G > A 561 G > A 588 T > G 621 C > T 633 G > A	666 G > A 724 T > C 777 T > G 1068 A > C 1158 T > C 1204 C > T 1443 T > C 1491 T > C
6) *S*.Kedougou Factory	0	1503	1491	78 G > A 555 G > A 579 G > T 588 T > G 633 G > A 666 G > A	972 A > T 978 A > G 1068 A > C 1159 T > C 1326 T > C 1368 T > C
7)*S*.Schwarzengrund factory	0	1503	1482	303 C > A 348 C > T 396 G > A 405 G > A 502 C > T 516 T > C 525 A > G 561 G > A 588 T > G	620 C > T 632 G > A 777 T > G 1068 A > C 1297 A > G 1336 T > C 1443 T > C 1460 A > G 1491 T > C
8)*S*.Schwarzengrund veterinary	0	1503	1482	60 T > C 303 C > A 348 C > T 396 G > A 405 G > A 502 C > T 516 T > C 525 A > G 555 G > A 561 G > A 588 T > G	621 C > T 632 G > A 777 T > G 1068 A > C 1158 T > C 1297 A > G 1336 T > C 1443 T > C 1460 A > G 1491 T > C

Table 28: Lists the SNP's observed in the *proP* gene across the eight isolates of *Salmonella.* The *proP gene* contains 1503 nucleotides and there were no gaps in any of the isolates. Multiple SNP's were seen across the gene in the eight isolates with identical SNP's visualised in the three S.Schwarzengrund isolates. These SNP's were explored in the translated amino acid BLAST to identify if the substitutions resulted in an amino acid change.

Figure 99 Translated amino acid pileup of the prop gene sequence across eight isolates of *Salmonella*

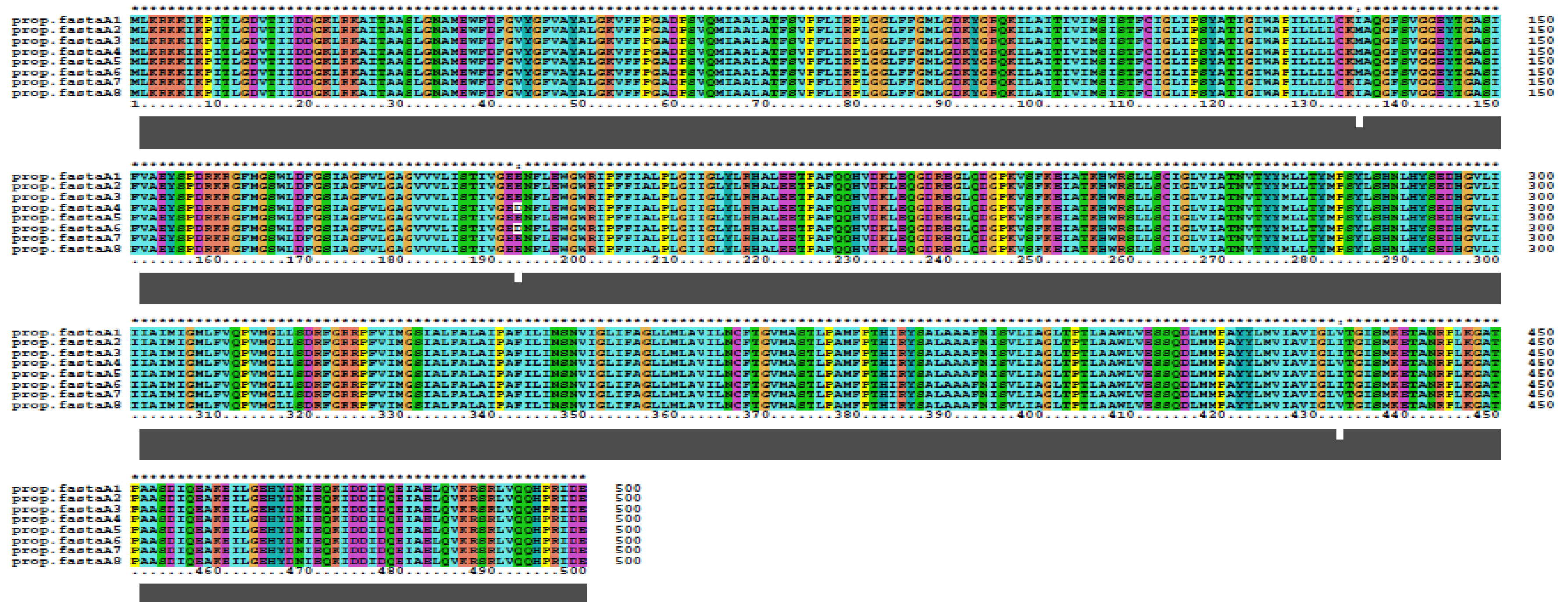


Figure 99: represents a translated pileup showing the 500 amino acids in the *proP* gene. Single nucleotide polymorphisms (SNP) can be observed in the clinical, factory and veterinary isolates of *S*.Schwarzengrund at around position 137 whereby amino acid M (Methionine) is substituted with amino acid I (Isoleucine). Then at around position 194 in 4 (*S*.Senftenberg 775W) and 6 (*S*.Kedougou factory), E (Glutamate) is substituted with D (Aspartate). Lastly, at around position 433, in 1 (*S*.Schwarzengrund FSL S5-458 American clinical), 4 (*S*.Senftenberg 775W), 7 (*S*.Schwarzengrund factory) and 8 (*S*.Schwarzengrund veterinary) the amino acid I (Isoleucine) is substituted with amino acid V (Valine),

7.4 Discussion

The aim of this chapter was to determine if there were any differences at the genomic level that may contribute to the persistence of the factory isolates of *Salmonella*, to reveal these differences a panel of eight isolates of *Salmonella* were submitted for sequencing. The data were then explored to search for differences in genome size and the possession of survival genes that may give the factory isolates an environmental advantage. Overall the results indicated that all the genes searched for were conserved across the eight *Salmonella* isolates, with only a few changes observed in the translated pileups. This is important as it revealed there were no major differences at the genomic level, to explain the persistence observed in the factory isolates.

The evolution of bacterial genomes may occur through a range of processes such as mutations, rearrangements or horizontal gene transfer. Research from sequencing projects has indicated that bacterial genomes not only code for key metabolic genes but also possess accessory genes that have been acquired by horizontal gene transfer which encode adaptive traits that may play a key role for bacteria under certain growth or environmental conditions (Juhas *et al.*, 2009). Comparing the bacterial genome size of the isolates provides a basic indication of whether the isolates had acquired any extra genetic information. Generally the genome size of *Salmonella* is reported as ~4.7 to 4.9 megabases (Chiu *et al.*, 2005; Allard *et al.*, 2013). The genome size of the factory isolates showed variation and ranged between 4.69 to 4.91mb. The largest genome was illustrated in *S*.Senftenberg 775W (5.32mb) and the veterinary isolate of *S*.Senftenberg (5.03mb). This vast difference in genome size indicates that these isolates of *Salmonella* are likely to have acquired genetic information through horizontal gene transfer. However this acquisition of extra genetic information appears to be serotype specific as neither of the largest genomes is of factory isolates. Other studies have also suggested that the genome size of *Salmonella* varies amongst serovars (Allard *et al.*, 2013; Andino and Hanning, 2015). Grépinet *et al.* (2012), sequenced *S*.Senftenberg SS209, a strain that is well documented as persistent in poultry. They reported a genome size of 5.02mb as well as a similar G+C content to our results for *S*.Senftenberg (51.73%) (Grépinet *et al.*, 2012). The genome size of the three isolates of *S*.Schwarzengrund ranged between 4.5mb to 4.7mb, with a G+C content of 51-52%, similarly Georgiades & Raoult (2011) reported the genome size of *S*.Schwarzengrund NC 011094 as 4.7mb with a 52% G+C content (Georgiades and Raoult, 2011).

Similarly, although Chiu *et al.* (2005) compared the genomes of different strains than those in the selected panel (*S.*Choleraesuis, *S.*Typhimurium LT2 and *S.*Typhi CT18), the findings of the study were similar and showed that despite the notable differences in pathogenicity of the three strains, overall the genomes were of similar size, indicating that the acquisition of genes had been counterbalanced by deletions as genes offering little overall selective benefit were lost rapidly (Chiu *et al.*, 2005).

Furthermore, other studies have suggested that adaptation to an environment is not necessarily linked to an increased genome size. The proposed theory states that non-specialised bacteria in communities frequently exchange genes and are referred to as `pre-species', however through evolution they specialise into different niches and as a result a decrease in gene exchanges occurs as well as a change in the overall repertoire of genes. Therefore, this indicates that the specialization of organisms (adaptation to environment) causes gene loss and inevitably the loss of regulation genes. This deregulation ultimately causes uncontrolled multiplication, and pathogenicity is demonstrated by destruction of the organisms' ecosystem. Their study compared the genomes of 12 different pathogenic bacteria and results indicated that specialized, pathogenic bacteria have smaller genomes in comparison to non-specialized bacteria. Thus, indicating that obtaining information about virulence factors is not sufficient in solely establishing an organism's pathogenic capacity, instead it is more valuable to study the repertoire of genes an organism possesses instead of searching for individual genes (Georgiades and Raoult, 2011). The accessibility of genomic microarrays allows the study of global regulatory networks and suites of genes (Taboada *et al.*, 2007).

Due to time constraint and resources it was not possible to conduct detailed analysis on each of the six genes selected, however the presence or absence was noted in the eight isolates and any SNP's that were observed were described. It was unable to determine whether these changes at the nucleotide and amino acid level would actually affect the function of the translated protein as this would require more detailed investigation.

Many bacteria including *S.*Typhimurium, use glycogen as a major energy storage compound as it contains glucose units. Glycogen is widely available in *Salmonella*, therefore it is reasonable to assume that under nutritional deprivation and suboptimal conditions, an increase of energy storage compounds like glycogen would be of importance (McMeechan *et al.*, 2005). The results from the current study suggested that the *glgC* gene was conserved across all eight isolates of *Salmonella* with only one

substitution noted at position 210 for the clinical, veterinary and factory isolates of *S.*Schwarzengrund. This is unlikely to result in variation in the functionality of the protein. Other studies have investigated the role of glycogen production in virulence, colonization and environmental survival of different *Salmonella enterica* serotypes. Other findings have suggested that although 17 of the 19 serotypes were positive for glycogen production, the role of glycogen in virulence and colonisation is minimal however in survival it has more significant impact with the *glgC* mutant surviving significantly less in both faeces and water at 4°C when the strain was grown in LB broth containing 0·5% glucose, in addition results in saline also showed a rapid decline after 7 days (McMeechan *et al.*, 2005). Furthermore, other investigations have emphasised that *Enterobacteria* have more than one important source of ADP glucose linked to glycogen biosynthesis. The use of *Escherichia coli* and *Salmonella* mutants which had the Δ*glgCAP* deleted and therefore lacked the whole glycogen biosynthetic machinery revealed that Δ*glgCAP* cells possessed many other proteins for catalysing the conversion of glucose-1-phosphate into ADP glucose (Morán-Zorzano *et al.*, 2007). Although the *glgC* gene may play a key role in survival, it is unlikely that the possession of the *glgC* gene solely is resulting in an environmental advantage for the factory isolates as the gene was conserved across all the isolates.

fur (ferric uptake regulator) is an important global regulator that acts in response to iron and regulates genes that encode iron transport systems, virulence factors and metabolic enzymes (Ikeda *et al.*, 2005; Somerville and Proctor, 2009). *Salmonella* responds to low pH through the acid tolerance response (ATR) and *fur* is implicated in ATR as it is acid sensitive and expresses acid shock proteins (Hall and Foster, 1996). *Fur* also activates *hilA* transcription which is a regulator of the *Salmonella* pathogenicity island 1 (SPI1) structural genes. The invasion of the epithelial cells is mediated by the type three secretory system which is encoded on SPI1 (Ellermeier and Slauch, 2008). The results from the current study suggest that the *fur* gene was conserved across all eight isolates of *Salmonella* and although multiple SNP's were observed across the nucleotide sequence none of these resulted in any point mutation of substitutions in the amino acid sequence.

hilA is an important global regulator that works as a transcriptional regulator for SPI1, SPI4 and SPI5 which collectively mediate host cell invasion (Lucas *et al.*, 2000; Garai *et al.*, 2012). The results from the current study suggest that the *hilA* gene was conserved across all eight isolates of *Salmonella*, yet multiple SNP's were seen across the nucleotide sequence in the eight isolates with identical SNP's visualised in the three *S.*Schwarzengrund isolates. The translated amino acid BLAST showed that these changes resulted in two SNP in the amino acid sequence; Firstly at around position 250 the three *S.*Schwarzengrund isolates showed a SNP whereby Isoleucine was substituted with Valine. Secondly at position 453, in the factory isolates of *S.*Livingstone and the veterinary isolate *S.*Senftenberg veterinary, the amino acid Leucine is replaced with Isoleucine. It is unlikely that these substitutions would have a large impact on protein function as all these amino acids are non-polar and hydrophobic, they fall into the aliphatic category, meaning the side chain contains only hydrogen and carbon atoms. These side chains are usually very non-reactive, and are therefore rarely involved directly in protein function, however they are involved in binding/recognition of hydrophobic ligands such as lipids (Betts and Russell, 2003).

Notable outbreaks associated with low moisture and desiccated foods have occurred in the past. Results in the survival chapter showed that *Salmonella* could survive on stainless surfaces for up to 35 days. Finn *et al.* (2013b), investigated the response of *S.* Typhimurium to desiccation on a stainless steel surface and to subsequent rehydration and showed 266 genes were expressed under desiccation stress in comparison to static broth culture. Drying up regulated the osmoprotectant transporters *proP*, *proU*, and *osmU*. Importantly, loss of any one of the three transport systems resulted in a reduction in the long-term viability of *S.*Typhimurium on stainless steel. In mutants where the *proP* gene was deleted, survival on stainless steel was lower and undetectable after 4 weeks (Finn *et al.*, 2013b). The *proP* gene was present in all eight isolates of *Salmonella.* Multiple SNP's were seen across the nucleotide sequence and these coded for 3 substitutions across the length of the amino acid sequence.

The first SNP was observed in the clinical, factory and veterinary isolates of *S.*Schwarzengrund at around position 137 whereby amino acid Methionine was substituted with amino acid Isoleucine. This is likely to be caused by a serotype difference rather than an environmental effect. Both methionine and isoleucine are hydrophobic amino acids; the methionine side chain is highly unreactive and is rarely involved in protein function. In line with other hydrophobic amino acids both are involved in binding/recognition of hydrophobic ligands such as lipids, one difference

is that methionine contains sulphur atom connected to a methyl group which is linked to bindings to atoms like metals, it limits the roles methionine can play in protein function. Isoleucine is also Cβ branched, most amino acids contain only one non-hydrogen substituent attached to their Cβ carbon, these amino acids contain two, this restricts isoleucine in the conformations the main chain can adopt (Betts and Russell, 2003).

The second substitution was observed at around position 194 in *S.*Senftenberg 775W and the factory isolate of *S.*Kedougou, whereby Glutamate was substituted with Aspartate. Both these amino acids are polar and are found on the surface of proteins, exposed to an aqueous environment. They are also involved in protein active or binding sites and having a negative charge means that they can interact with positively-charged non-protein atoms, such as cations like zinc. Aspartate is the preferred amino acid in protein active sites as its side chain is shorter than that of than glutamate; this means it is more rigid within protein structures (Betts and Russell, 2003). Lastly, at around position 433, in all three isolates of *S.*Schwarzengrund and *S.*Senftenberg 775W, Isoleucine was substituted with amino acid Valine, as discussed above these are both non-reactive aliphatic chains that are rarely linked to protein function.

OmpR is a transcriptional regulatory protein that is needed for the expression of the outer membrane protein genes *ompF* and *ompC.* It reacts to changes in osmolality and regulates invasion in addition to being involved in intracellular survival (Bang *et al.*, 2000; Walthers *et al.*, 2003). The *ompR* gene also plays a pivotal role in biofilm formation. Dong *et al.* (2011) found that *ompR* mutants were unable to produce cellulose and curli both of which are crucial for biofilm formation (Dong *et al.*, 2011). The *OmpR* gene was conserved across all eight isolates of *Salmonella.* Multiple SNP's were seen across the nucleotide sequence however these did not code for any point mutations or substitutions across the length of the amino acid sequence.

It is well recognised that bacteria are able to persist in response to environmental stresses. The *RpoS* gene encodes for the *RpoS* alternative sigma factor which is a global regulator that regulates approximately 50 genes in response to environmental stress or during entry into stationary phase. The regulation of *Rpos* is complex and involves multiple factors. Some examples of stress factor which initiate *RpoS* regulation includes; acid shock, heat shock and a shortage of carbon, nitrogen and

phosphate sources (Ibanez-Ruiz *et al.*, 2000; Brown *et al.*, 2002; Dodd and Aldsworth, 2002).

Results indicate the *rpoS gene* was conserved across the eight *Salmonella* isolates, while only a few SNP's were observed in the nucleotide sequence, all three isolates of *S.*Schwarzengrund had the same number of SNP's at the same position. These SNP's translated into four SNP's in the amino acid sequence. Firstly at around position 110 in the factory isolate of *S.*Livingstone, the factory isolate of *S.*Senftenberg factory and the veterinary isolate of *S.*Senftenberg, Threonine was substituted with Isoleucine. Threonine is commonly found in protein functional centres. The hydroxyl group is reactive and can form hydrogen bonds with many polar substrates. Although isoleucine is more hydrophobic compared with threonine, they both share a common feature in that they are *C-beta* branched, most amino acids contain only one non-hydrogen substituent attached to their C-beta carbon, whereas valine, threonine and isoleucine all encompass two, meaning they are limited in the conformations the main-chain can form (Betts and Russell, 2003).

The second SNP was observed at around position 143 in the factory isolate of *S.*Livingstone where Leucine was substituted with Serine. Being a hydrophobic amino acid, leucine prefers to be hidden in protein hydrophobic cores. It is also more inclined to be within alpha helices more so than in beta strands. Serine is an indifferent amino acid that can be found either within the interior of a protein or on the protein surface, it is frequently found in protein functional centres. The hydroxyl group is reactive and can form hydrogen bonds with many polar substrates.

The third SNP was observed in the clinical, factory and veterinary isolates of *S.*Schwarzengrund, at position 220, whereby Serine is substituted with Phenylalanine. Serine is a small amino acid that can be found both inside and on the surface of a protein, it contains a reactive hydroxyl group that can form hydrogen bonds with a variety of polar substrates. Phenylalanine is usually found in the protein hydrophobic core, it contains an aromatic side chain but is relatively unreactive rarely directly involved in protein function. However, it does have a role in substrate recognition and interactions with non-protein ligands consisting of aromatic groups through stacking interaction. The fourth SNP was also observed in these three *S.*Schwarzengrund isolates at position 272, whereby Alanine was substituted with Valine. Alanine is a simple amino acid in that it is neither hydrophobic nor polar and is non-reactive, and thus rarely directly involved in protein function. It is involved in substrate recognition

or specificity, particularly in interactions with other non-reactive atoms such as carbon. Valine is a hydrophobic amino acid but is also non-reactive and therefore has limited role in protein fiction (Betts and Russell, 2003).

7.5 Conclusion

When compared to the reference genome, the results indicated that there were some notable differences in genome size and SNP's in genes across the *Salmonella* isolates, these are likely to be confined to serotype rather than an effect of environment as many identical SNP's were observed across the serotype matched isolates of *S*.Schwarzengrund. The nucleotide and amino acid sequences of the food factory isolates were similar to those of isolates from other environments and no major genomic rearrangements were observed to indicate that the factory isolates had any fundamental genomic variations. These genomic data supports the phenotypic and metabolic analysis in that there were no profound genomic differences associated with the factory isolates. Further data mining of the genomic sequence data is required to reveal any potential differences that may be associated with the factory isolates.

8 Final discussion

Globally the burden of *Salmonella* is estimated at 93.8 million cases annually, of which a staggering 80.3 million are thought to be associated with foodborne illness (Majowicz *et al.*, 2010). In the UK, *Salmonella* is a leading cause of bacterial foodborne infection, second only to *Campylobacter* (Majowicz *et al.*, 2010; Newell *et al.*, 2010). Although reports have suggested a steady decline in the number of cases of *Salmonella* infections over the past 10 years, which is likely to be attributed to a greater awareness of food safety and hygiene principles and vaccination of egg laying hens, the rates of *Salmonella* infections still remain high across Europe, which is a concern for public health (EFSA and ECDC, 2015).

Strategies have already been implemented to prevent the spread of pathogens at the food manufacturing level, however epidemiological data suggests that *Salmonella* is able to persist in the food manufacturing environments and cause subsequent cross-contamination of food products (Holah and Lelieveld, 2011). One of the difficulties faced by the industry is that cross- contamination of food products with *Salmonella* at the factory level is a multifactorial process that is difficult to manage. It involves the management of the organisms entering the food factory on raw materials, the accumulation of organisms which may become `resident', the design of the food factory in terms of equipment and surfaces used, in addition to the role of personnel. It has been suggested that the persistence of bacteria in food manufacturing environments may be linked to resident strains becoming more resistant as they are able adapt to environmental stresses, whereas the counterargument is that strains are not being addressed adequately with a properly implemented food safety and hygiene management system.

This study aimed to characterise isolates of *Salmonella*, known to be persistent in the food manufacturing environment, by comparing their microbiological characteristics with a panel of matched clinical and veterinary isolates, the intention being to characterise effective control strategies in the manufacture of food products.

Therefore, in the first experimental chapter, the isolates in the challenge panel were phenotypically characterised in order to determine any gross morphological differences characteristic of the strains from each environment. The results revealed all the isolates of *Salmonella* were motile and the strains isolated from the factory environment shared common phenotypic characteristics with those from human clinical and veterinary environments. The API profiles of *Salmonella* from the three groups were identical and did not demonstrate any differences across the strains, with the exception of *S*.Senftenberg which was negative for production of hydrogen sulphide, a characteristic of this strain which is well recognised (Henry *et al.*, 1969; Yi *et al.*, 2014). Analysis of cell length as revealed by SEM did not reveal any significant differences in the gross morphology of the cells and all the cell lengths were within expected parameters. These results were in line with findings from other studies that have reported the rod lengths of *Salmonella* and *L.monocytogenes* (Harshey and Matsuyama, 1994; Batt and Robinson, 1999; Motarjemi, 2013; Andino and Hanning, 2015).

Previously, Mattick *et al.* (2000) investigated the morphological changes in isolates of *Salmonella at* reduced A_w values and revealed that the *Salmonella* strains tested formed filaments at both 21°C and 37°C. The presence of filamentation in low-A_w food products could pose serious implications for the food industry, as contaminant *Salmonella* cells may reveal low counts via traditional microbiological methods such as direct plating or enrichment. However, under favourable conditions the presence of a large number of viable *Salmonella* cells may cause infection following consumption (Mattick *et al.*, 2000).

Organisms evolve a metabolic profile which is more closely mapped to the environment in which they exist: they express genes required if certain reagents or substrates are available in order to make optimal use of their metabolic system (Gibson, 2008; Seshasayee *et al.*, 2009). A more detailed analysis of the metabolism of the *Salmonella* strains was undertaken in the second experimental chapter using a high resolution phenotypic microarray (Biolog Inc. Microbial Identification Systems GEN III). The results showed that a majority of *Salmonella* strains isolated from the food factory environment shared a common metabolic capacity and were able to use a similar diversity of organic nutrients to the human clinical strains and veterinary isolates. Absorbance data in combination with PCA analysis allowed for the discrimination of the control organism, *L.monocytogenes,* on the basis of its distinct metabolic profile; this was expected as *L.monocytogenes* is a different genus and has an expected different metabolic profile. The data also highlighted the differences in metabolism of the factory isolate of *S*.Montevideo, irrespective of the environment it was isolated from, it was distinct from other salmonellae on the basis of a differential metabolic ability to utilise glucose and the sugar alcohol group more effectively than the other *Salmonella* isolates.

This type of analysis allowed for exploratory data mining of complex data to reveal patterns within that data that might be discriminatory or characteristic of underlying features such as an organism's ability to utilise a different carbon source or have a different resistance profile. On the basis of this experimental chapter the distinctive clustering of the remainder of the *Salmonella* isolates, including those that were serotype- matched showed that the isolates were metabolically indistinguishable. Therefore, the ability to utilize carbon sources and differences in chemical sensitivity profiles are unlikely to be a sole contributory factor to *Salmonella* persistence in the food factory environment.

A possible extension of this experimental work could be to investigate the relationship between the unregulated metabolites identified by metabolic microarrays. More in depth data mining using the KEGG pathway database will allow further investigation of pathways that may lead to enhanced environmental adaptation, as well as identifying a pool of candidate genes involved in pathways.

The persistence of *Salmonella* on steel surfaces is also of great concern to the food industry as they may serve as a focus for cross- contamination of food product. The third experimental chapter sought to identify parameters known to influence bacterial survival in the food factory environment; and to define the effect of environmental factors such as temperature and humidity. The ability of food factory strains of *Salmonella* to demonstrate an enhanced growth rate in nutrient rich and deficient media was investigated at 10°C, 25°C and 37°C. In addition to survival of *Salmonella* on stainless steel coupons at 10°C, 25°C and 37°C with varying Relative Humidity (RH) levels. Once the cells could no longer be cultivated on nutrient agar, the ability of the strains to enter the VBNC phase was explored.

The results from this chapter indicated that *Salmonella* survival on stainless steel was affected by environmental temperatures that may be experienced in a food processing environment; with higher survival rates at temperatures close to 25°C and lower humidity levels of 15% RH, and a rapid decline in cell count with lower temperatures of 10°C and higher humidity of 70% RH and at 37°C with 20% humidity. Also, several resident factory strains survived in higher numbers on stainless steel compared to serotype matched clinical and veterinary isolates at both 25°C and 10°C. Furthermore, enhanced survival was not serotype specific; at 10°C the food factory isolate of *S*.Seftenberg demonstrated the longest D value (D_{10}=6.1) and *S*.Senftenberg 775W demonstrate the shortest D value (D_{10}=2.2). These findings were supported by observations by others investigating the survival of *Salmonella* on surfaces (Chaitiemwong *et al.*, 2010; Habimana *et al.*, 2010b; Margas *et al.*, 2013).

The fluorescence microscopy images of samples stained with Baclight supported the microbiology data at 37°C, 25°C and 10°C, however at 10°C when the cfu data was <10 cfu ml^{-1}, the results revealed that the factory, clinical and veterinary isolates of *S*.Schwarzengrund were possibly entering the VBNC state. This state may be induced by stress, as *Salmonella* is primarily a foodborne pathogen; this may demonstrate a huge risk to food manufacturers and the public. Compared to normal culturable cells, VBNC cells have only lost the ability to grow on routine agar (Oliver, 2005; Li *et al.*, 2014), evidence suggests that the cell membrane is actually intact and cells contain undamaged genetic information (Heidelberg *et al.*, 1997; Cook and Bolster, 2007). Unlike dead cells, VBNC cells are metabolically active and are able to carry out respiration, utilize nutrients and convert amino acids into proteins (Li *et al.*, 2014). Most importantly, the fact that cells can be resuscitated insinuates the possibility for cell growth and re-infection (Waldner *et al.*, 2012; Li *et al.*, 2014).

To survive and persist in the food factory environment it is reasonable to assume that *Salmonella* isolates might be able to grow and establish themselves more readily in nutrient depreciated conditions. Across all temperatures and media both nutritious and nutrient limited, the factory isolates of *Salmonella* did not show any competitive fitness advantage and grew in concordance with serotype-matched strains from clinical and veterinary environments. In addition, the results also indicated that in minimal M9 salt medium the factory isolates did not show enhanced growth relative to other serotypes, therefore growth is unlikely to be a major factor driving *Salmonella* persistence.

Therefore, undertaking further investigations to understand the interplay between temperature and humidity levels and modelling conditions in the food factory environment to identify areas where *Salmonella* can harbour may play an important role in eliminating the organism from food processing environments. In the current study samples were stored statically at a set temperature/ humidity, cycling the temperature and humidity levels observed in the food factory may differentiate the food isolates; it could be that the factory isolates are able to better adapt to environmental changes in comparison to serotype- matched isolates from clinical and veterinary sources. This could be achieved through the incubation of samples in an environmental chamber that cycles programmed temperature and humidity levels over a period of time.

Microorganisms are able to survive on food processing surfaces and in the food industry this is clearly problematic. Studies have shown that the persistence of *Salmonella* is correlated to its ability to form biofilms (Habimana *et al.*, 2010b; Kostaki *et al.*, 2012). The ability of bacteria to form biofilms has sparked research interest for many organisations from the food safety management perspective (Vestby *et al.*, 2009b; Vestby *et al.*, 2009a; Shi and Zhu, 2009), especially as biofilms are difficult to control as they may form in areas of factory plants where cleaning is difficult (Djordjevic *et al.*, 2002). Biofilms have serious implications in the food industry as the detachment of cells in biofilm can lead to cross contamination of food products, causing spoilage as well as the transmission of infectious disease. Therefore in the fourth experimental chapter the biofilm formation capacity of the panel of *Salmonella* and *Listeria monocytogenes* isolates at different temperatures and times in both nutrient rich and nutrient deprived media was established.

The results from the current study highlighted that all the isolates in the challenge panel were able to form biofilms in both nutritious and nutrient limited environments and temperature played an important role, with higher levels of biofilm production occurring at 25°C and 37°C. At 37°C an extended duration of incubation had no beneficial effect on the ability of strains to form more established biofilms however at 25°C, 15°C and 10°C more established biofilm formation was correlated with increased incubation of 48 hours. None of the factory isolates showed an enhanced capability to form biofilms in comparison to serotype-matched isolates from veterinary and clinical sources. However, irrespective of media and temperature, only *S.*Senftenberg 775W produced biofilms at levels almost twice as high as other isolates in the panel. *S.*Senftenberg 775W is a known heat resistant strain and is used as a model organism in the food industry (Goepfert and Biggie, 1968; Ng *et al.*, 1969). Therefore it is unlikely that biofilm formation in isolation is responsible for the environmental persistence observed in the

food isolates, however it is likely to play a contributory factor in other persistence strategies such as resistance to biocides, desiccation or other environmental extremes.

In the current study, the biofilm forming ability of pure cultures of *Salmonella* isolated from factory, veterinary and clinical environments was investigated and they were all found to produce biofilm; it was important to bench mark the biofilm producing capabilities of the panel of isolates as pure cultures prior to more in depth investigation. It is recognised that the factory micro-biome is likely to contain competing organisms that are coexisting with *Salmonella* therefore further investigation could include identifying other organisms isolated from the manufacturing environment and studying the synergistic capacity of *Salmonella* to from biofilms with these competing organisms. Other research has suggested that a synergistic effect may have an influence on biofilm persistence. Habimana *et al.* (2010b) showed that biofilms of *S*. Agona were supported in a mixed species biofilm with both *Pseudomonas* species and *Staphylococcus* spp. (Habimana *et al.*, 2010b).

Cells in biofilm are more difficult to eradicate as they have a diversity of defence mechanisms and unlike in their planktonic state the cells are more resistant to antimicrobials (Costerton *et al.*, 1995a; Moretro *et al.*, 2009a). Therefore, understanding the relationship between *Salmonella* biofilms and sensitivity to disinfectants is crucial in the control of bacterial contamination for the food industry. The fifth experimental chapter determined the susceptibility of the panel of isolates to a range of disinfectants typically used in the food industry, in addition to determining the ability of these disinfectants to penetrate through two day mature biofilms grown in micro titre plates. As anticipated the results revealed that planktonic cells were more susceptible to disinfectants than *Salmonella* cells in a biofilm. Secondly, although all the disinfectants tested were successful in reducing the bacterial load none completely eradicated cells in the biofilm even with increased contact time. Applying disinfectants for the manufacturers recommended contact time was essential when removing cells in biofilm as overall a shorter contact time of approximately one minute did not achieve the desired levels of cell reduction.

Investigating the speed with which resistance may be acquired through repeated sub-lethal exposure may be of importance Some of the isolates will already have been pre-exposed to many of the chemical agents tested in the current study, which could explain the lower cell reduction observed for Tego 2000 and Virkon, both of these disinfectants are commonly used in the food industry and as mentioned above, studies have shown that environmental strains exposed to repeated sub lethal concentrations of disinfectants can lead to resistance (Braoudaki and Hilton, 2004; Condell *et al.*, 2012).To prevent resistance to agents it is important that plants frequently rotate the disinfectants used.

Importantly this chapter revealed that all of the chemicals tested were effective against the factory isolates of *Salmonella* and with sufficient contact times a majority of the agents were able to exhibit a $\geq 4 \log_{10}$ reduction in cell number for cells in biofilm. The chemicals used and protocols vary from plant to plant and these data suggest further investigation analysing the protocols, information about contact times and the adherence to protocols would be fundamental for the control of microorganisms in the food manufacturing environment.

In the investigations discussed in previous chapters, the factory isolates as a group did not show any enhanced phenotypic, morphological or survival advantages that may lead to their persistence. However it was hypothesised there may be differences at the genomic level which were not immediately expressed as measurable traits. Therefore the aim of the final experimental chapter was to determine if there were any gross differences at the genomic level that may contribute to the persistence of the factory isolates of *Salmonella*. To reveal these differences a panel of eight isolates of *Salmonella* were subject to whole genome sequencing. The data were then mined to explore for differences in genome size and the possession of survival genes that may give the factory isolates an environmental advantage. When compared to the reference sequenced genome, the results indicated that there were some notable differences in genome size and SNP's in genes across the *Salmonella* isolates, *these* were *likely* to be confined to serotype rather than an effect of environment, as many identical SNPs were observed across the serotype matched isolates of *S*.Schwarzengrund. The nucleotide and amino acid sequences of the food factory isolates were similar to those of isolates from other environments and no major genomic rearrangements were observed to indicate horizontal gene transfer. This genomic study supports the phenotypic and metabolic analysis in that there were no profound genomic differences associated with the factory isolates.

This chapter focussed on exploring descriptive genomics to reveal any gross genomic differences across the panel of isolates. Further, detailed bioinformatics of the genome sequence would be helpful in revealing the potential differences that may be associated with the factory isolates. It would be anticipated that any significant changes observed at this gross genomic level that would have had an impact at the phenotypic level and would have been highlighted in the phenotypic microarrays. However, the phenotypic microarray data revealed no differences in the metabolism of the factory isolates of *Salmonella* that were sequenced. There may be other transcriptional or regulatory networks that are different and are not associated with metabolism that may have been missed by the phenotypic microarrays and may be revealed through genomic microarrays.

In conclusion, having investigated a variety of morphological, biochemical and genomic factors, it is unlikely that the persistence of *Salmonella* in the food manufacturing environment is attributable to a single phenotypic, metabolic or genomic factor. Whilst a combination of microbiological factors may be involved it is also possible that strain persistence in the factory environment is a consequence of failure to apply established hygiene management principles.

9 References

ABDI, H. & WILLIAMS, L. J. 2010. Principal component analysis. *Wiley Interdisciplinary Reviews: Computational Statistics,* 2**,** 433-459.

ADAK, G. K., LONG, S. M. & O'BRIEN, S. J. 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut,* 51**,** 832-41.

ADAMS, M. R. & MOSS, M. O. 2000. *Food Microbiology.2nd edition.,* Cambridge, Royal Society of Chemistry.

AL-FATTANI, M. A. & DOUGLAS, L. J. 2004. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrobial Agents and Chemotherapy,* 48**,** 3291-3297.

ALDRIDGE, K. E., GARDNER, B. B., CLARK, S. J. & MATSEN, J. M. 1978. Comparison of Micro-ID, API 20E, and Conventional Media Systems in Identification of Enterobacteriaceae. *Journal of Clinical Microbiology,* 7**,** 507-513.

ALIKHAN, N.-F., PETTY, N., BEN ZAKOUR, N. & BEATSON, S. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics,* 12**,** 402.

ALLARD, M. W., LUO, Y., STRAIN, E., PETTENGILL, J., TIMME, R., WANG, C., LI, C., KEYS, C. E., ZHENG, J., STONES, R., WILSON, M. R., MUSSER, S. M. & BROWN, E. W. 2013. On the Evolutionary History, Population Genetics and Diversity among Isolates of Salmonella Enteritidis PFGE Pattern JEGX01.0004. *PLoS ONE,* 8**,** e55254.

ANDINO, A. & HANNING, I. 2015. Salmonella enterica: Survival, Colonization, and Virulence Differences among Serovars. *The Scientific World Journal,* 2015**,** 520179.

APEL, D., WHITE, A. P., GRASSL, G. A., FINLAY, B. B. & SURETTE, M. G. 2009. Long-term survival of Salmonella enterica serovar Typhimurium reveals an infectious state that is underrepresented on laboratory media containing bile salts. *Appl Environ Microbiol,* 75**,** 4923-5.

ARAÚJO, P., A, LEMOS, M., MERGULHÃO, F., MELO, L. & SIMÕES, M. 2013. The Influence of Interfering Substances on the Antimicrobial Activity of Selected Quaternary Ammonium Compounds. *International Journal of Food Science,* 2013**,** 9.

ARNOLD, J. W. & SILVERS, S. 2000. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. *Poult Sci,* 79**,** 1215-21.

ASCENZI, J. M. 1995. *Handbook of Disinfectants and Antiseptics*, Taylor & Francis.

BAKER, C. G. J., RANKEN, D. & KILL, R. C. 2012. *Food Industries Manual*, Springer US.

BANERJEE, M. & SARKAR, P. K. 2004. Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. *Food Microbiology,* 21**,** 335-342.

BANG, I. S., KIM, B. H., FOSTER, J. W. & PARK, Y. K. 2000. OmpR Regulates the Stationary-Phase Acid Tolerance Response of Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology,* 182**,** 2245-2252.

BARROW, P. A., LOVELL, M. A. & BARBER, L. Z. 1996. Growth suppression in early-stationary-phase nutrient broth cultures of Salmonella typhimurium and Escherichia coli is genus specific and not regulated by sigma S. *Journal of Bacteriology,* 178**,** 3072-3076.

BASF, T. C. C. 2013. Sorgene 5 The tried and trusted broad-spectrum disinfectant for the best livestock hygiene and farm biosecurity. Cheshire, UK.

BATT, C. A. & ROBINSON, R. K. 1999. *Encyclopedia of Food Microbiology*, Elsevier Science.

BAUERMEISTER, L. J., BOWERS, J. W. J., TOWNSEND, J. C. & MCKEE, S. R. 2008. The Microbial and Quality Properties of Poultry Carcasses Treated with Peracetic Acid as an Antimicrobial Treatment. *Poultry Science,* 87**,** 2390-2398.

BAUMGARTNER, R., RYNER, L., RICHTER, W., SUMMERS, R., JARMASZ, M. & SOMORJAI, R. 2000. Comparison of two exploratory data analysis methods for fMRI: fuzzy clustering vs. principal component analysis. *Magn Reson Imaging,* 18**,** 89-94.

BBC. 1988. *1988: Egg industry fury over salmonella claim* [Online]. Available: http://news.bbc.co.uk/onthisday/hi/dates/stories/december/3/newsid_2519000/2519451.stm [Accessed 07/12/2014.

BD, B. D. A. C. 2011. BBL™ SIM Medium L007503.

BEHRAVESH, C. B., FERRARO, A., DEASY, M., 3RD, DATO, V., MOLL, M., SANDT, C., REA, N. K., RICKERT, R., MARRIOTT, C., WARREN, K., URDANETA, V., SALEHI, E., VILLAMIL, E., AYERS, T., HOEKSTRA, R. M., AUSTIN, J. L., OSTROFF, S. & WILLIAMS, I. T. 2010. Human Salmonella infections linked to contaminated dry dog and cat food, 2006-2008. *Pediatrics,* 126**,** 477-83.

BELL, C. & KYRIAKIDES, A. 2002. *Salmonella: A practical approach to the organism and its control in foods,* Oxford, Blackwell Science.

BERNEY, M., HAMMES, F., BOSSHARD, F., WEILENMANN, H.-U. & EGLI, T. 2007. Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. *Applied and Environmental Microbiology,* 73**,** 3283-3290.

BETANCOR, L., SCHELOTTO, F., MARTINEZ, A., PEREIRA, M., ALGORTA, G., RODRÍGUEZ, M. A., VIGNOLI, R. & CHABALGOITY, J. A. 2004. Random Amplified Polymorphic DNA and Phenotyping Analysis of Salmonella enterica Serovar Enteritidis Isolates Collected from Humans and Poultry in Uruguay from 1995 to 2002. *Journal of Clinical Microbiology,* 42**,** 1155-1162.

BETTS, M. J. & RUSSELL, R. B. 2003. Amino Acid Properties and Consequences of Substitutions. *Bioinformatics for Geneticists.* John Wiley & Sons, Ltd.

BIOLOG 2008. GEN III MicroPlateTM, Instructions for Use , Not for human in vitro diagnostic use. Hayward, CA.

BLOCK, S. S. 2001. *Disinfection, Sterilization, and Preservation*, Lippincott Williams & Wilkins.

BOCHNER, B. R. 2009. Global phenotypic characterization of bacteria. *Fems Microbiology Reviews,* 33**,** 191-205.

BOWDEN, S. D., ROWLEY, G., HINTON, J. C. D. & THOMPSON, A. 2009. Glucose and Glycolysis Are Required for the Successful Infection of Macrophages and Mice by Salmonella enterica Serovar Typhimurium. *Infection and Immunity,* 77**,** 3117-3126.

BRANDS, D. A., ALCAMO, I. E. & HEYMANN, D. L. 2009. *Salmonella*, Facts On File, Incorporated.

BRAOUDAKI, M. 2004. *Antibiotic and biocide resistaance in Salmonella enterica and Escherichia coli 0157.*, Aston University

BRAOUDAKI, M. & HILTON, A. C. 2004. Adaptive Resistance to Biocides in Salmonella enterica and Escherichia coli O157 and Cross-Resistance to Antimicrobial Agents. *Journal of Clinical Microbiology,* 42**,** 73-78.

BREMER, P. J., MONK, I. & OSBORNE, C. M. 2001a. Survival of <i>Listeria monocytogenes</i> Attached to Stainless Steel Surfaces in the Presence or Absence of <i>Flavobacterium</i> spp. *Journal of Food Protection,* 64, 1369-1376.

BREMER, P. J., MONK, I. & OSBORNE, C. M. 2001b. Survival of Listeria monocytogenes attached to stainless steel surfaces in the presence or absence of Flavobacterium spp. *J Food Prot,* 64, 1369-76.

BRENNER, F. W., VILLAR, R. G., ANGULO, F. J., TAUXE, R. & SWAMINATHAN, B. 2000. Salmonella Nomenclature. *Journal of Clinical Microbiology,* 38, 2465-2467.

BROOKS, B. W., PERRY, M. B., LUTZE-WALLACE, C. L. & MACLEAN, L. L. 2008. Structural characterization and serological specificities of lipopolysaccharides from Salmonella enterica serovar Gallinarum biovar Pullorum standard, intermediate and variant antigenic type strains. *Veterinary Microbiology,* 126, 334-344.

BROWN, L., GENTRY, D., ELLIOTT, T. & CASHEL, M. 2002. DksA Affects ppGpp Induction of RpoS at a Translational Level. *Journal of Bacteriology,* 184, 4455-4465.

BUCKINGHAM-MEYER, K., GOERES, D. M. & HAMILTON, M. A. 2007. Comparative evaluation of biofilm disinfectant efficacy tests. *Journal of microbiological methods,* 70, 236-244.

BUZOLEVA, L. S. & CHUMAK, A. D. 2000. Metabolism of Poly-β-Hydroxybutyric Acid in Yersinia pseudotuberculosis and Listeria monocytogenes Cultivated at Different Temperatures. *Microbiology,* 69, 652-654.

CABEÇA, T. K., PIZZOLITTO, A. C. & PIZZOLITTO, E. L. 2012. Activity of disinfectants against foodborne pathogens in suspension and adhered to stainless steel surfaces. *Brazilian Journal of Microbiology,* 43, 1112-1119.

CÁNOVAS, D., FLETCHER, S. A., HAYASHI, M. & CSONKA, L. N. 2001. Role of Trehalose in Growth at High Temperature of Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology,* 183, 3365-3371.

CARRASCO, E., MORALES-RUEDA, A. & GARCÍA-GIMENO, R. M. 2012. Cross-contamination and recontamination by Salmonella in foods: A review. *Food Research International,* 45, 545-556.

CDA, C. D. A. 2012. Antimicrobial Copper FAQs. Hempstead, UK: CDA.

CDC, C. F. D. C. A. P. 2009. Multistate Outbreak of Salmonella Infections Associated with Peanut Butter and Peanut Butter–Containing Products -United States, 2008–2009. January 29, 2009 ed.

CHAITIEMWONG, N., HAZELEGER, W. C. & BEUMER, R. R. 2010. Survival of Listeria monocytogenes on a conveyor belt material with or without antimicrobial additives. *International Journal of Food Microbiology,* 142, 260-263.

CHIU, C.-H., TANG, P., CHU, C., HU, S., BAO, Q., YU, J., CHOU, Y.-Y., WANG, H.-S. & LEE, Y.-S. 2005. The genome sequence of Salmonella enterica serovar Choleraesuis, a highly invasive and resistant zoonotic pathogen. *Nucleic Acids Research,* 33, 1690-1698.

CHRISTISON, C. A., LINDSAY, D. & VON HOLY, A. 2007. Cleaning and Handling Implements as Potential Reservoirs for Bacterial Contamination of Some Ready-to-Eat Foods in Retail Delicatessen Environments. *Journal of Food Protection,* 70, 2878-2883.

CHUANCHUEN, R., PATHANASOPHON, P., KHEMTONG, S., WANNAPRASAT, W. & PADUNGTOD, P. 2008. Susceptibilities to Antimicrobials and Disinfectants in <i>Salmonella</i> Isolates Obtained from Poultry and Swine in Thailand. *Journal of Veterinary Medical Science,* 70, 595-601.

CLEMENTS, M., ERIKSSON, S., TEZCAN-MERDOL, D., HINTON, J. C. D. & RHEN, M. 2001. Virulence gene regulation in Salmonella enterica. *Annals of Medicine,* 33**,** 178-185.
COGAN, T. A. & HUMPHREY, T. J. 2003. The rise and fall of Salmonella Enteritidis in the UK. *Journal of Applied Microbiology,* 94**,** 114-119.
CONDELL, O., IVERSEN, C., COONEY, S., POWER, K. A., WALSH, C., BURGESS, C. & FANNING, S. 2012. Efficacy of Biocides Used in the Modern Food Industry To Control Salmonella enterica, and Links between Biocide Tolerance and Resistance to Clinically Relevant Antimicrobial Compounds. *Applied and Environmental Microbiology,* 78**,** 3087-3097.
COOK, K. L. & BOLSTER, C. H. 2007. Survival of Campylobacter jejuni and Escherichia coli in groundwater during prolonged starvation at low temperatures. *J Appl Microbiol,* 103**,** 573-83.
CORCORAN, M., MORRIS, D., DE LAPPE, N., O'CONNOR, J., LALOR, P., DOCKERY, P. & CORMICAN, M. 2014. Commonly used disinfectants fail to eradicate Salmonella enterica biofilms from food contact surface materials. *Appl Environ Microbiol,* 80**,** 1507-14.
COSTERTON, J., LEWANDOWSKI, Z., CALDWELL, D., KORBER, D. & LAPPINSCOTT, H. 1995a. Microbial biofilms. *Annu Rev Microbiol,* 49**,** 711 - 745.
COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995b. Microbial biofilms. *Annu Rev Microbiol,* 49**,** 711-45.
DARBANDI, F. 2010. *Parallel Comparison of Accuracy in Vitek2 Auto analyzer and API 20 E / API 20 NE Microsystems*

Master of Science with a Major in Chemical engineering Masters, University College of Borås.
DAWSON, P., HAN, I., COX, M., BLACK, C. & SIMMONS, L. 2007. Residence time and food contact time effects on transfer of Salmonella Typhimurium from tile, wood and carpet: testing the five-second rule. *J Appl Microbiol,* 102**,** 945-53.
DI BONAVENTURA, G., PICCOLOMINI, R., PALUDI, D., D'ORIO, V., VERGARA, A., CONTER, M. & IANIERI, A. 2008. Influence of temperature on biofilm formation by Listeria monocytogenes on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology,* 104**,** 1552-1561.
DJORDJEVIC, D., WIEDMANN, M. & MCLANDSBOROUGH, L. A. 2002. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation. *Applied and Environmental Microbiology,* 68**,** 2950-2958.
DODD, C. E. & ALDSWORTH, T. G. 2002. The importance of RpoS in the survival of bacteria through food processing. *Int J Food Microbiol,* 74**,** 189-94.
DONG, H., PENG, D., JIAO, X., ZHANG, X., CHEN, S., LU, Y., GENG, S. & LIU, X. 2011. [Construction and characterization of an ompR gene deletion mutant from Salmonella enteritidis]. *Wei Sheng Wu Xue Bao,* 51**,** 1256-62.
DONLAN, R. M. 2002. Biofilms: microbial life on surfaces. *Emerg Infect Dis,* 8**,** 881-90.
DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews,* 15**,** 167-193.
DUPONT 2009. Virkon®S Disinfectant and Virucide. USA.
EFSA, E. F. S. A. & ECDC, E. C. F. D. P. A. C. 2012. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010. . *EFSA Journal 2012,* 10**,** 442.

EFSA, E. F. S. A. & ECDC, E. C. F. D. P. A. C. 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *European Food Safety Authority Journal,* 13**,** 1-162.

EISSA, M. E., EL NABY KAMEL, M. A. & BESHIR, M. 2014. Study of Antimicrobial Power of Amphoteric Disinfectants of Tego Series Used in Pharmaceutical Industry. *Egyption Academic Journal of Biological Sciences Microbiology,* 6**,** 17-27.

EL-SHENAWY, M. A. & MARTH, E. H. 1990. Behavior of Listeria monocytogenes in the presence of gluconic acid and during preparation of cottage cheese curd using gluconic acid. *J Dairy Sci,* 73**,** 1429-38.

ELAMIN, A. 2006. *Cadbury apologises for Salmonella outbreak* [Online]. William Reed Buisness media. Available: http://www.foodproductiondaily.com/Safety-Regulation/Cadbury-apologises-for-Salmonella-outbreak [Accessed 07/12/2014.

ELLERMEIER, J. R. & SLAUCH, J. M. 2008. Fur Regulates Expression of the Salmonella Pathogenicity Island 1 Type III Secretion System through HilD. *Journal of Bacteriology,* 190**,** 476-486.

EURO-INOX, E. S. S. D. A. 2006. Stainless Steel in the Food and Beverage Industry. *In:* PARTINGTON, E. (ed.) *Materials and Applications Series.* First ed. Brussels.

FAUNDEZ, G., TRONCOSO, M., NAVARRETE, P. & FIGUEROA, G. 2004. Antimicrobial activity of copper surfaces against suspensions of Salmonella enterica and Campylobacter jejuni. *BMC Microbiology,* 4**,** 19.

FAURE, D. 2002. The Family-3 Glycoside Hydrolases: from Housekeeping Functions to Host-Microbe Interactions. *Applied and Environmental Microbiology,* 68**,** 1485-1490.

FAZLARA, A. & EKHTELAT, M. 2012. The Disinfectant Effects of Benzalkonium Chloride on Some Important Foodborne Pathogens. *American-Eurasian Journal of Agriculture & Environmental Science* 12**,** 23-29.

FERREIRA, V., BARBOSA, J., STASIEWICZ, M., VONGKAMJAN, K., MORENO SWITT, A., HOGG, T., GIBBS, P., TEIXEIRA, P. & WIEDMANN, M. 2011. Diverse Geno- and Phenotypes of Persistent Listeria monocytogenes Isolates from Fermented Meat Sausage Production Facilities in Portugal. *Applied and Environmental Microbiology,* 77**,** 2701-2715.

FINN, S., CONDELL, O., MCCLURE, P., AMÉZQUITA, A. & FANNING, S. 2013a. Mechanisms of survival, responses and sources of Salmonella in low-moisture environments. *Frontiers in Microbiology,* 4**,** 331.

FINN, S., HÄNDLER, K., CONDELL, O., COLGAN, A., COONEY, S., MCCLURE, P., AMÉZQUITA, A., HINTON, J. C. D. & FANNING, S. 2013b. ProP Is Required for the Survival of Desiccated Salmonella enterica Serovar Typhimurium Cells on a Stainless Steel Surface. *Applied and Environmental Microbiology,* 79**,** 4376-4384.

FINNEGAN, M., LINLEY, E., DENYER, S. P., MCDONNELL, G., SIMONS, C. & MAILLARD, J.-Y. 2010. Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. *Journal of Antimicrobial Chemotherapy,* 65**,** 2108-2115.

FRAENKEL, D., OSBORN, M. J., HORECKER, B. L. & SMITH, S. M. 1963. Metabolism and cell wall structure of a mutant of salmonella typhimurium deficient in phosphoglucose isomerase. *Biochemical and Biophysical Research Communications,* 11**,** 423-428.

FREHEL, C., HELLIO, R., CREMIEUX, A.-C., CONTREPOIS, A. & BOUVET, A. 1988. Nutritionally variant streptococci develop ultrastructural abnormalities during experimental endocarditis. *Microbial Pathogenesis,* 4**,** 247-255.

FSA, F. S. A. 2008. Management of outbreaks of foodborne illness in England and Wales. London: Food Standards Agency

FSA, F. S. A. 2011. London. Available: http://www.food.gov.uk/news-updates/news/2011/4809/eggs [Accessed 07/12/2014.
GANDHI, M. & CHIKINDAS, M. L. 2007. Listeria: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology,* 113**,** 1-15.
GANDHI, M. & MATTHEWS, K. R. 2003. Efficacy of chlorine and calcinated calcium treatment of alfalfa seeds and sprouts to eliminate Salmonella. *Int J Food Microbiol,* 87**,** 301-6.
GARAI, P., GNANADHAS, D. P. & CHAKRAVORTTY, D. 2012. Salmonella enterica serovars Typhimurium and Typhi as model organisms: Revealing paradigm of host-pathogen interactions. *Virulence,* 3**,** 377-388.
GARRETT, T. R., BHAKOO, M. & ZHANG, Z. 2008. Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science,* 18**,** 1049-1056.
GEHAN, M., ANWER, W., AMER, H. M., EL-SABAGH, I. M., REZK, A. & BADAWY, E. M. 2009. In vitro Efficacy Comparisons of Disinfectants Used inthe Commercial Poultry Farms. *International Journal of Poultry Science* 8**,** 237-241.
GEORGIADES, K. & RAOULT, D. 2011. Genomes of the Most Dangerous Epidemic Bacteria Have a Virulence Repertoire Characterized by Fewer Genes but More Toxin-Antitoxin Modules. *PLoS ONE,* 6**,** e17962.
GIANNELLA, R. A. 1996. Medical Microbiology. 4th edition. Galveston University of Texas Medical Branch at Galveston.
GIAOURIS, E., NYCHAS, G. J., CHORIANOPOULOS, N. & SKANDAMIS, P. 2012. *Attachment and Biofilm Formation by Salmonella in Food Processing Environments,* Greece, INTECH Open Access Publisher.
GIBSON, G. 2008. The environmental contribution to gene expression profiles. *Nat Rev Genet,* 9**,** 575-581.
GIERALTOWSKI, L., JULIAN, E., PRINGLE, J., MACDONALD, K., QUILLIAM, D., MARSDEN-HAUG, N., SAATHOFF-HUBER, L., VON STEIN, D., KISSLER, B., PARISH, M., ELDER, D., HOWARD-KING, V., BESSER, J., SODHA, S., LOHARIKAR, A., DALTON, S., WILLIAMS, I. & BEHRAVESH, C. B. 2013. Nationwide outbreak of Salmonella Montevideo infections associated with contaminated imported black and red pepper: warehouse membership cards provide critical clues to identify the source. *Epidemiology and Infection,* 141**,** 1244-1252.
GILBRETH, S. E., BENSON, A. K. & HUTKINS, R. W. 2004. Catabolite Repression and Virulence Gene Expression in Listeria monocytogenes. *Current Microbiology,* 49**,** 95-98.
GILLMAIER, N., GOTZ, A., SCHULZ, A., EISENREICH, W. & GOEBEL, W. 2012. Metabolic responses of primary and transformed cells to intracellular Listeria monocytogenes. *PLoS One,* 7**,** e52378.
GNN, G. N. N. 2004. *How does DNA sequencing work?* [Online]. J. Craig Venter Institute. Available: http://www.genomenewsnetwork.org/resources/whats_a_genome/Chp2_2.shtml [Accessed 20/05/2015.
GOEPFERT, J. M. & BIGGIE, R. A. 1968. Heat resistance of Salmonella typhimurium and Salmonella senftenberg 775W in milk chocolate. *Applied Microbiology,* 16**,** 1939-1940.
GORMLEY, F. J., LITTLE, C. L., RAWAL, N., GILLESPIE, I. A., LEBAIGUE, S. & ADAK, G. K. 2011. A 17-year review of foodborne outbreaks: describing the continuing decline in England and Wales (1992-2008). *Epidemiol Infect,* 139**,** 688-99.
GRÉPINET, O., BOUMART, Z., VIRLOGEUX-PAYANT, I., LOUX, V., CHIAPELLO, H., GENDRAULT, A., GIBRAT, J.-F., CHEMALY, M. & VELGE, P. 2012.

Genome Sequence of the Persistent Salmonella enterica subsp. enterica Serotype Senftenberg Strain SS209. *Journal of Bacteriology,* 194**,** 2385-2386.
GROSSMAN, E., REITER, G., STURZENBERGER, O. P., DE LA ROSA, M., DICKINSON, T. D., FLRRETTI, G. A., LUDLAM, G. E. & MECKEL, A. H. 1986. Six-month study of the effects of a chlorhexidine mouthrinse on gingivitis in adults. *Journal of Periodontal Research,* 21**,** 33-43.
GUERIN, P. J., DE JONG, B., HEIR, E., HASSELTVEDT, V., KAPPERUD, G., STYRMO, K., GONDROSEN, B., LASSEN, J., ANDERSSON, Y. & AAVITSLAND, P. 2004. Outbreak of Salmonella Livingstone infection in Norway and Sweden due to contaminated processed fish products. *Epidemiol Infect,* 132**,** 889-95.
HABIMANA, O., HEIR, E., LANGSRUD, S., ASLI, A. & MORETRO, T. 2010a. Enhanced surface colonization of Escherichia coli O157:H7 in biofilms of an Acinetobacter calcoaceticus isolate from meat-processing environments. *Appl Environ Microbiol,* 76**,** 4557 - 4559.
HABIMANA, O., MORETRO, T., LANGSRUD, S., VESTBY , L., NESSE, L. & HEIR, E. 2010b. Micro ecosystems from feed industry surfaces: a survival and biofilm study of Salmonella versus host resident flora strains. *BMC Veterinary Research,* 6**,** 48.
HALL, H. K. & FOSTER, J. W. 1996. The role of fur in the acid tolerance response of Salmonella typhimurium is physiologically and genetically separable from its role in iron acquisition. *J Bacteriol,* 178**,** 5683-91.
HAMED, S. J., AL-YASIRI, I. L., ALI, N. T. & AL-FERON, M. A. 2014. Antibacterial Activity of Calcium Hydroxide Combined with Chlrohexidine or Sodium Hypochlorite against Gram Positive and Gram Negative Bacteria *Journal of Natural Sciences Research,* 4**,** 55-61.
HARDY, A. 2004. Salmonella: a continuing problem. *Postgraduate Medical Journal,* 80**,** 541-545.
HARSHEY, R. M. & MATSUYAMA, T. 1994. Dimorphic transition in Escherichia coli and Salmonella typhimurium: surface-induced differentiation into hyperflagellate swarmer cells. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 8631-8635.
HASMAN, H., SAPUTRA, D., SICHERITZ-PONTEN, T., LUND, O., SVENDSEN, C. A., FRIMODT-MØLLER, N. & AARESTRUP, F. M. 2014. Rapid Whole-Genome Sequencing for Detection and Characterization of Microorganisms Directly from Clinical Samples. *Journal of Clinical Microbiology,* 52**,** 139-146.
HEIDELBERG, J. F., SHAHAMAT, M., LEVIN, M., RAHMAN, I., STELMA, G., GRIM, C. & COLWELL, R. R. 1997. Effect of aerosolization on culturability and viability of gram-negative bacteria. *Appl Environ Microbiol,* 63**,** 3585-8.
HENRY, N., BAYNE, H. G. & GARIBALDI, J. A. 1969. Heat Resistance of Salmonella: the Uniqueness of Salmonella senftenberg 775W. *Applied Microbiology,* 17**,** 78-82.
HERIKSTAD, H., MOTARJEMI, Y., TAUXE, R. & NBSP, V. 2002. Salmonella surveillance: a global survey of public health serotyping. *Epidemiology & Infection,* 129**,** 1-8.
HEYNDRICKX, M., VANDEKERCHOVE, D., HERMAN, L., ROLLIER, I., GRIJSPEERDT, K. & DE ZUTTER, L. 2002. Routes for salmonella contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiol Infect,* 129**,** 253-65.
HINE, E. A., STEFEN, E. K. & WAGNER, J. E. 1988. New semisolid agar for the detection of motile salmonellae. *Journal of Clinical Microbiology,* 26**,** 875-878.
HOLAH, J. & LELIEVELD, H. 2011. *Hygienic Design of Food Factories*, Elsevier Science.

HPA, H. P. A. 2009. Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods. London.
HUGAS, M. & BELOEIL, P. 2014. Controlling Salmonella along the food chain in the European Union - progress over the last ten years. Italy.
HUMPHREY, T. J., SLATER, E., MCALPINE, K., ROWBURY, R. J. & GILBERT, R. J. 1995. Salmonella enteritidis phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. *Applied and Environmental Microbiology,* 61**,** 3161-3164.
HURRELL, E., KUCEROVA, E., LOUGHLIN, M., CAUBILLA-BARRON, J. & FORSYTHE, S. J. 2009. Biofilm formation on enteral feeding tubes by Cronobacter sakazakii, Salmonella serovars and other Enterobacteriaceae. *Int J Food Microbiol,* 136**,** 227-31.
IBANEZ-RUIZ, M., ROBBE-SAULE, V., HERMANT, D., LABRUDE, S. & NOREL, F. 2000. Identification of RpoS (ς(S))-Regulated Genes in Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology,* 182**,** 5749-5756.
ICMSF, I. C. O. M. S. F. F. 1996. *Microorganisms in Foods 5: Characteristics of Microbial Pathogens*, Springer.
IKEDA, J. S., JANAKIRAMAN, A., KEHRES, D. G., MAGUIRE, M. E. & SLAUCH, J. M. 2005. Transcriptional Regulation of sitABCD of Salmonella enterica Serovar Typhimurium by MntR and Fur. *Journal of Bacteriology,* 187**,** 912-922.
IMEN, B. S., MZOUGHI RIDHA, M. & MAHJOUB, A. 2012. *Laboratory Typing Methods for Diagnostic of Salmonella Strains, the "Old" Organism That Continued Challenges, Salmonella - A Dangerous Foodborne Pathogen,* Tunisia, In-Tech.
INTERVET 2002. Fighting salmonella? Get the killer punch! Netherlands.
INTERVET 2004. poultry focus; business news for the poultry industry. *Poultry Focus.* May 2004 ed. Milton Keynes: Intervet.
INTERVET. 2014. *Salmonella control - United Kingdom* [Online]. Available: http://www.safe-poultry.com/SalmonellacontrolUK.asp [Accessed 07/12/2014.
IOANNOU, C. J., HANLON, G. W. & DENYER, S. P. 2007. Action of Disinfectant Quaternary Ammonium Compounds against Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 51**,** 296-306.
IOWA-STATE, U. 2015. *How the SEM Works* [Online]. Available: http://www.mse.iastate.edu/research/laboratories/sem/microscopy/how-does-the-sem-work/high-school/how-the-sem-works/ [Accessed 11/01/2015.
IWAMOTO, M., AYERS, T., MAHON, B. E. & SWERDLOW, D. L. 2010. Epidemiology of Seafood-Associated Infections in the United States. *Clinical Microbiology Reviews,* 23**,** 399-411.
JANG, K. I., KIM, M. G., HA, S. D., KIM, K. S., LEE, K. H., CHUNG, D. H., KIM, C. H. & KIM, K. Y. 2007. Morphology and adhesion of Campylobacter jejuni to chicken skin under varying conditions. *J Microbiol Biotechnol,* 17**,** 202-6.
JAY, L., D.DAVOS, DUNDAS, M., FRANKISH, E. & LIGHTFOOT, D. 2003. Salmonella. *Hocking AD (ed) Foodborne microorganisms of public health significance. 6th ed,* . Sydney: Australian Institute of Food Science and Technology (NSW Branch).
JAYARAO, B. M., DONALDSON, S. C., STRALEY, B. A., SAWANT, A. A., HEGDE, N. V. & BROWN, J. L. 2006. A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in pennsylvania. *J Dairy Sci,* 89**,** 2451-8.
JOHNSON, F. H. & SCHWARZ, H. W. 1944. Carbohydrate Utilization by Hydrocarbon Bacteria. *Journal of Bacteriology,* 47**,** 373-378.

JOLLIFFE, I. 2014. Principal Component Analysis. *Wiley StatsRef: Statistics Reference Online*. John Wiley & Sons, Ltd.
JORDAN, E. O., CALDWELL, M. E. & REITER, D. 1934. Bacterial Motility. *J Bacteriol,* 27**,** 165-74.
JOSEPH, B. & GOEBEL, W. 2007. Life of Listeria monocytogenes in the host cells' cytosol. *Microbes and Infection,* 9**,** 1188-1195.
JOSEPH, B., OTTA, S., KARUNASAGAR, I. & KARUNASAGAR, I. 2001. Biofilm formation by Salmonella spp. on food contact surfaces and their sensitivity to sanitizers. *Int J Food Microbiol,* 64**,** 367 - 372.
JUHAS, M., VAN DER MEER, J. R., GAILLARD, M., HARDING, R. M., HOOD, D. W. & CROOK, D. W. 2009. Genomic islands: tools of bacterial horizontal gene transfer and evolution. *Fems Microbiology Reviews,* 33**,** 376-393.
KALÁB, M., YANG, A. F. & CHABOT, D. 2008. Conventional Scanning Electron Microscopy of Bacteria. *Journal of Microscopy***,** 44-60.
KANEHISA LABORATORIES 2010. KEGG Pathway Maps.
KAPPERUD, G., GUSTAVSEN, S., HELLESNES, I., HANSEN, A. H., LASSEN, J., HIRN, J., JAHKOLA, M., MONTENEGRO, M. A. & HELMUTH, R. 1990. Outbreak of Salmonella typhimurium infection traced to contaminated chocolate and caused by a strain lacking the 60-megadalton virulence plasmid. *J Clin Microbiol,* 28**,** 2597-601.
KELLETT, B. S. 1979. Bacterial growth in amphylobactic disinfectant solution. . *Laboratory Animals,* 13, **,** 135–138.
KIM, K. Y. & FRANK, J. F. 1994. Effect of Growth Nutrients on Attachment of <i>Listeria monocytogenes</i> To Stainless Steel. *Journal of Food Protection,* 57**,** 720-724.
KIM, W. & SURETTE, M. G. 2004. Metabolic differentiation in actively swarming Salmonella. *Molecular Microbiology,* 54**,** 702-714.
KITIS, M. 2004. Disinfection of wastewater with peracetic acid: a review. *Environment International,* 30**,** 47-55.
KLEE, S. R., ÖZEL, M., APPEL, B., BOESCH, C., ELLERBROK, H., JACOB, D., HOLLAND, G., LEENDERTZ, F. H., PAULI, G., GRUNOW, R. & NATTERMANN, H. 2006. Characterization of Bacillus anthracis-Like Bacteria Isolated from Wild Great Apes from Côte d'Ivoire and Cameroon. *Journal of Bacteriology,* 188**,** 5333-5344.
KOSTAKI, M., CHORIANOPOULOS, N., BRAXOU, E., NYCHAS, G.-J. & GIAOURIS, E. 2012. Differential Biofilm Formation and Chemical Disinfection Resistance of Sessile Cells of Listeria monocytogenes Strains under Monospecies and Dual-Species (with Salmonella enterica) Conditions. *Applied and Environmental Microbiology,* 78**,** 2586-2595.
KRAUSE, M., UKKONEN, K., HAATAJA, T., RUOTTINEN, M., GLUMOFF, T., NEUBAUER, A., NEUBAUER, P. & VASALA, A. 2010. A novel fed-batch based cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. *Microbial Cell Factories,* 9**,** 11.
KUMAR, C. G. & ANAND, S. K. 1998. Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology,* 42**,** 9-27.
KUMAR, S. 2012. *Textbook of Microbiology*, Jaypee Brothers, Medical Publishers.
KUSUMANINGRUM, H. D., RIBOLDI, G., HAZELEGER, W. C. & BEUMER, R. R. 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology,* 85**,** 227-236.
LAMETSCHWANDTNER, A., LAMETSCHWANDTNER, U. & WEIGER, T. 1990. Scanning electron microscopy of vascular corrosion casts--technique and applications: updated review. *Scanning microscopy,* 4**,** 889-940; discussion 941.

LANGSRUD, S., MØRETRØ, T. & SUNDHEIM, G. 2003. Characterization of Serratia marcescens surviving in disinfecting footbaths. *Journal of Applied Microbiology,* 95**,** 186-195.
LE HELLO, S., BRISABOIS, A., ACCOU-DEMARTIN, M., JOSSE, A., MARAULT, M., FRANCART, S., DA SILVA, N. J. & WEILL, F.-X. 2012. Foodborne Outbreak and Nonmotile Salmonella enterica Variant, France. *Emerging Infectious Diseases,* 18**,** 132-134.
LE HELLO, S., HARROIS, D., BOUCHRIF, B., SONTAG, L., ELHANI, D., GUIBERT, V., ZEROUALI, K. & WEILL, F. X. 2013. Highly drug-resistant Salmonella enterica serotype Kentucky ST198-X1: a microbiological study. *Lancet Infect Dis,* 13**,** 672-9.
LEE WONG, A. C. 1998. Biofilms in Food Processing Environments. *Journal of Dairy Science,* 81**,** 2765-2770.
LEIFSON, E. 1951. STAINING, SHAPE, AND ARRANGEMENT OF BACTERIAL FLAGELLA. *Journal of Bacteriology,* 62**,** 377-389.
LEWIS, M. J. & STOCKER, B. A. D. 1971. A biochemical subdivision of one phage type of Salmonella typhimurium. *Epidemiology & Infection,* 69**,** 683-691.
LI, L., MENDIS, N., TRIGUI, H., OLIVER, J. D. & FAUCHER, S. P. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in Microbiology,* 5**,** 258.
LINDBÄCK, T., ROTTENBERG, M. E., ROCHE, S. M. & RØRVIK LIV, M. 2010. The ability to enter into an avirulent viable but non-culturable (VBNC) form is widespread among Listeria monocytogenes isolates from salmon, patients and environment. *Veterinary Research,* 41**,** 08.
LITTLE, C. L., RICHARDSON, J. F., OWEN, R. J., DE PINNA, E. & THRELFALL, E. J. 2008. Campylobacter and Salmonella in raw red meats in the United Kingdom: Prevalence, characterization and antimicrobial resistance pattern, 2003–2005. *Food Microbiology,* 25**,** 538-543.
LIU, D. 2006. Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. *Journal of Medical Microbiology,* 55**,** 645-659.
LIU, JANET Z., JELLBAUER, S., POE, A. J., TON, V., PESCIAROLI, M., KEHL-FIE, T. E., RESTREPO, NICOLE A., HOSKING, M. P., EDWARDS, ROBERT A., BATTISTONI, A., PASQUALI, P., LANE, THOMAS E., CHAZIN, WALTER J., VOGL, T., ROTH, J., SKAAR, ERIC P. & RAFFATELLU, M. 2012. Zinc Sequestration by the Neutrophil Protein Calprotectin Enhances Salmonella Growth in the Inflamed Gut. *Cell Host & Microbe,* 11**,** 227-239.
LUCAS, R. L., LOSTROH, C. P., DIRUSSO, C. C., SPECTOR, M. P., WANNER, B. L. & LEE, C. A. 2000. Multiple Factors Independently Regulate hilA and Invasion Gene Expression in Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology,* 182**,** 1872-1882.
LUNDEN, J., MIETTINEN, M., AUTIO, T. & KORKEALA, H. 2000. Persistent Listeria monocytogenes strains show enhanced adherence to food contact surface after short contact times. *J Food Prot,* 63**,** 1204 - 1207.
LUSCOMBE, N. M., GREENBAUM, D. & GERSTEIN, M. 2001. What is bioinformatics? A proposed definition and overview of the field. *Methods Inf Med,* 40**,** 346-58.
MAH, T. F. & O'TOOLE, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol,* 9**,** 34-9.
MAJOWICZ, S. E., MUSTO, J., SCALLAN, E., ANGULO, F. J., KIRK, M., O'BRIEN, S. J., JONES, T. F., FAZIL, A. & HOEKSTRA, R. M. 2010. The global burden of nontyphoidal Salmonella gastroenteritis. *Clin Infect Dis,* 50**,** 882-9.

MANIJEH, M., MOHAMMAD, J. & ROHA, K. K. 2008. Biofilm Formation by Salmonella Spp. on Food Contact Surfaces and their Sensitivity to Sanitizers. *International Journal of Food Microbiology,* 8**,** 367–372.

MARCUS, S. L., BRUMELL, J. H., PFEIFER, C. G. & FINLAY, B. B. 2000. Salmonella pathogenicity islands: big virulence in small packages. *Microbes and Infection,* 2**,** 145-156.

MARGAS, E., ALSTROM-MOOREA, A., DODD, C. & HOLAHA, J. 2013. Salmonella survival in low aw environment. 1-6.

MATTICK, K. L., JORGENSEN, F., LEGAN, J. D., COLE, M. B., PORTER, J., LAPPIN-SCOTT, H. M. & HUMPHREY, T. J. 2000. Survival and filamentation of Salmonella enterica serovar enteritidis PT4 and Salmonella enterica serovar typhimurium DT104 at low water activity. *Appl Environ Microbiol,* 66**,** 1274-9.

MCCARROLL., M. 2008. *Application of the BIOLOG microplate system to monitor the physiological response of heat-stressed bacteria.* Master of Science in Environmental Microbiology, West Virginia University.

MCDONNELL, G. & RUSSELL, A. D. 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews,* 12**,** 147-179.

MCMEECHAN, A., LOVELL, M. A., COGAN, T. A., MARSTON, K. L., HUMPHREY, T. J. & BARROW, P. A. 2005. Glycogen production by different Salmonella enterica serotypes: contribution of functional glgC to virulence, intestinal colonization and environmental survival. *Microbiology,* 151**,** 3969-3977.

MCMULLAN, D. 1995. Scanning electron microscopy 1928–1965. *Scanning,* 17**,** 175-185.

MEAD, P. S., SLUTSKER, L., DIETZ, V., MCCAIG, L. F., BRESEE, J., SHAPIRO, C., GRIFFIN, P. M. & TAUXE, R. 1999. Food-Related Illness and Death in the United States. *Emerging Infectious Disease journal,* 5**,** 607.

MEYER-LUECKEL, H. & PARIS, S. 2013. *Caries Management - Science and Clinical Practice*, Thieme.

MILIOTIS, M. D. & BIER, J. W. 2003. *International Handbook of Foodborne Pathogens,* USA, CRC Press.

MISTRY, P. 2012. *Salmonella in companion animals.* Aston University

MITCHELL, W. J., REIZER, J., HERRING, C., HOISCHEN, C. & SAIER, M. H. 1993. Identification of a phosphoenolpyruvate:fructose phosphotransferase system (fructose-1-phosphate forming) in Listeria monocytogenes. *Journal of Bacteriology,* 175**,** 2758-2761.

MORÁN-ZORZANO, M. T., ALONSO-CASAJÚS, N., MUÑOZ, F. J., VIALE, A. M., BAROJA-FERNÁNDEZ, E., EYDALLIN, G. & POZUETA-ROMERO, J. 2007. Occurrence of more than one important source of ADPglucose linked to glycogen biosynthesis in Escherichia coli and Salmonella. *FEBS Letters,* 581**,** 4423-4429.

MORETRO, T. & LANGSRUD, S. 2004. Listeria monocytogenes : biofilm formation and persistence in food-processing environments. *Biofilms,* 1**,** 107 - 121.

MORETRO, T., MIDTGAARD, E., NESSE, L. & LANGSRUD, S. 2003. Susceptibility of Salmonella isolated from fish feed factories to disinfectants and air-drying at surfaces. *Vet Microbiol,* 94**,** 207 - 217.

MORETRO, T., VESTBY, L., NESSE, L., HANNEVIK, S., KOTLARZ, K. & LANGSRUD, S. 2009a. Evaluation of efficiency of disinfectants against Salmonella from the feed industry. *J Appl Microbiol,* 106**,** 1005 - 1012.

MORETRO, T., VESTBY, L., NESSE, L., STORHEIM, S., KOTLARZ, K. & LANGSRUD, S. 2009b. Evaluation of efficacy of disinfectants against Salmonella from the feed industry. *J Appl Microbiol,* 106**,** 1005 - 1012.

MOREY, A. & SINGH, M. 2012. Low-temperature survival of Salmonella spp. in a model food system with natural microflora. *Foodborne Pathogens and Disease,* 9**,** 218-23.

MORISHIGE, Y., TANDA, M., FUJIMORI, K., MINO, Y. & AMANO, F. 2014. Induction of viable but non-culturable (VBNC) state in Salmonella cultured in M9 minimal medium containing high glucose. *Biol Pharm Bull,* 37**,** 1617-25.

MOTARJEMI, Y. 2013. *Encyclopedia of Food Safety*, Elsevier Science.

MOTOSHIMA, M., YANAGIHARA, K., MORINAGA, Y., MATSUDA, J., HASEGAWA, H., KOHNO, S. & KAMIHIRA, S. 2012. Identification of bacteria directly from positive blood culture samples by DNA pyrosequencing of the 16S rRNA gene. *Journal of Medical Microbiology,* 61**,** 1556-1562.

MSEFULA, C. L., KINGSLEY, R. A., GORDON, M. A., MOLYNEUX, E., MOLYNEUX, M. E., MACLENNAN, C. A., DOUGAN, G. & HEYDERMAN, R. S. 2012. Genotypic Homogeneity of Multidrug Resistant S. Typhimurium Infecting Distinct Adult and Childhood Susceptibility Groups in Blantyre, Malawi. *PLoS ONE,* 7**,** e42085.

NASCUTIU, A. M. 2010. [Viable non-culturable bacteria]. *Bacteriol Virusol Parazitol Epidemiol,* 55**,** 11-8.

NESSE, L., NORDBY, K., HEIR, E., BERGSJOE, B., VARDUND, T. & NYGAARD, H. 2003. Molecular analyses of Salmonella enterica isolates from fish feed factories and fish feed ingredients. *Appl Environ Microbiol,* 69**,** 1075 - 1081.

NEWELL, D. G., KOOPMANS, M., VERHOEF, L., DUIZER, E., AIDARA-KANE, A., SPRONG, H., OPSTEEGH, M., LANGELAAR, M., THREFALL, J., SCHEUTZ, F., DER GIESSEN, J. V. & KRUSE, H. 2010. Food-borne diseases — The challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology,* 139, Supplement**,** S3-S15.

NG, H., BAYNE, H. G. & GARIBALDI, J. A. 1969. Heat Resistance of Salmonella: the Uniqueness of Salmonella senftenberg 775W. *Applied Microbiology,* 17**,** 78-82.

NGUYEN, H. D. N. & YUK, H.-G. 2013. Changes in resistance of Salmonella Typhimurium biofilms formed under various conditions to industrial sanitizers. *Food Control,* 29**,** 236-240.

NICKERSON, C. A. & CURTISS, R., 3RD 1997. Role of sigma factor RpoS in initial stages of Salmonella typhimurium infection. *Infect Immun,* 65**,** 1814-23.

NUCERA, D. M., MADDOX, C. W., HOIEN-DALEN, P. & WEIGEL, R. M. 2006. Comparison of API 20E and invA PCR for Identification of Salmonella enterica Isolates from Swine Production Units. *Journal of Clinical Microbiology,* 44**,** 3388-3390.

O'HARA, C. M., RHODEN, D. L. & MILLER, J. M. 1992. Reevaluation of the API 20E identification system versus conventional biochemicals for identification of members of the family Enterobacteriaceae: a new look at an old product. *Journal of Clinical Microbiology,* 30**,** 123-125.

O'HARA, C. M., TENOVER, F. C. & MILLER, J. M. 1993. Parallel comparison of accuracy of API 20E, Vitek GNI, MicroScan Walk/Away Rapid ID, and Becton Dickinson Cobas Micro ID-E/NF for identification of members of the family Enterobacteriaceae and common gram-negative, non-glucose-fermenting bacilli. *Journal of Clinical Microbiology,* 31**,** 3165-3169.

O'TOOLE, G. A. 2011. Microtiter Dish Biofilm Formation Assay. *Journal of Visualized Experiments : JoVE***,** 2437.

OLIVEIRA, K., OLIVEIRA, T., TEIXEIRA, P., AZEREDO, J. & OLIVEIRA, R. 2007. Adhesion of Salmonella Enteritidis to stainless steel surfaces. *Brazilian Journal of Microbiology,* 38**,** 318-323.

OLIVER, J. D. 2005. The viable but nonculturable state in bacteria. *J Microbiol,* 43 Spec No**,** 93-100.
OLSEN, J., HOEGH-ANDERSEN, K., CASADESUS, J. & THOMSEN, L. 2012. The importance of motility and chemotaxis for extra animal survival of Salmonella enterica serovar Typhimurium and Dublin. *J Appl Microbiol,* 113**,** 560 - 568.
OLSEN, J. E., HOEGH-ANDERSEN, K. H., CASADESUS, J., ROSENKRANTZ, J., CHADFIELD, M. S. & 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**,** 67.
PAIVA, D. M., SINGH. M, MACKLIN.K.S, PRICE.S.B, HESS .J.B & CONNER.D.E. 2009. Antimicrobial Activity of Commercial Concrete Sealant Against Salmonella Spp: A Model for Poultry Processing Plants. *International Journal of Poultry Science,* 8**,** 939-945.
PARKIN, A., BOWMAN, L., ROESSLER, M. M., DAVIES, R. A., PALMER, T., ARMSTRONG, F. A. & SARGENT, F. 2012. How Salmonella oxidises H2 under aerobic conditions. *FEBS Letters,* 586**,** 536-544.
PEELE, D., BRADFIELD, J., PRYOR, W. & VORE, S. 1997. Comparison of identifications of human and animal source gram-negative bacteria by API 20E and crystal E/NF systems. *Journal of Clinical Microbiology,* 35**,** 213-216.
PÉREZ-RODRÍGUEZ, F., VALERO, A., TODD, E., CARRASCO, E., GARCÍA-GIMENO, R. & ZURERA, G. 2007. Modeling transfer of Escherichia coli O157: H7 and Staphylococcus aureus during slicing of a cooked meat product. *Meat science,* 76**,** 692-699.
PERFUMO, A., ELSAESSER, A., LITTMANN, S., FOSTER, R. A., KUYPERS, M. M. M., COCKELL, C. S. & KMINEK, G. 2014. *Epifluorescence, SEM, TEM and nanoSIMS image analysis of the cold phenotype of Clostridium psychrophilum at subzero temperatures*.
PHLS, P. H. L. S. 1997. Preliminary report of an international outbreak of Salmonella anatum infection linked to an infant formula milk. *Euro Surveill,* 2**,** 22-24.
PINE, L., MALCOLM, G. B., BROOKS, J. B. & DANESHVAR, M. I. 1989. Physiological studies on the growth and utilization of sugars by Listeria species. *Can J Microbiol,* 35**,** 245-54.
PODOLAK, R., ENACHE, E., STONE, W., BLACK, D. G. & ELLIOTT, P. H. 2010. Sources and Risk Factors for Contamination, Survival, Persistence, and Heat Resistance of <i>Salmonella</i> in Low-Moisture Foods. *Journal of Food Protection,* 73**,** 1919-1936.
PREMARATNE, R. J., LIN, W. J. & JOHNSON, E. A. 1991. Development of an improved chemically defined minimal medium for Listeria monocytogenes. *Applied and Environmental Microbiology,* 57**,** 3046-3048.
PROUTY, A. M. & GUNN, J. S. 2003. Comparative Analysis of Salmonella enterica Serovar Typhimurium Biofilm Formation on Gallstones and on Glass. *Infection and Immunity,* 71**,** 7154-7158.
RAI, V. R. & BAI, J. A. 2014. *Microbial Food Safety and Preservation Techniques*, Taylor & Francis.
RAMESH, N., JOSEPH, S., CARR, L. E., DOUGLASS, L. & WHEATON, F. 2002. Evaluation of chemical disinfectants for the elimination of Salmonella biofilms from poultry transport containers. *Poultry Science,* 81**,** 904-910.
REINA-BUENO, M., ARGANDONA, M., SALVADOR, M., RODRIGUEZ-MOYA, J., IGLESIAS-GUERRA, F., CSONKA, L. N., NIETO, J. J. & VARGAS, C. 2012. Role of trehalose in salinity and temperature tolerance in the model halophilic bacterium Chromohalobacter salexigens. *PLoS One,* 7**,** e33587.

RENNER, L. D. & WEIBEL, D. B. 2011. Physicochemical regulation of biofilm formation. *MRS bulletin / Materials Research Society,* 36**,** 347-355.

ROBINSON, A., MCCARTER, Y. S. & TETREAULT, J. 1995. Comparison of Crystal Enteric/Nonfermenter system, API 20E system, and Vitek AutoMicrobic system for identification of gram-negative bacilli. *Journal of Clinical Microbiology,* 33**,** 364-370.

RODRIGUEZ-URREGO, J., HERRERA-LEON, S., ECHEITA-SARRIONDIA, A., SOLER, P., SIMON, F. & MATEO, S. 2010. Nationwide outbreak of Salmonella serotype Kedougou associated with infant formula, Spain, 2008. *Euro Surveill,* 15**,** 19582.

ROMERO, R., SCHAUDINN, C., KUSANOVIC, J. P., GORUR, A., GOTSCH, F., WEBSTER, P., NHAN-CHANG, C. L., EREZ, O., KIM, C. J., ESPINOZA, J., GONCALVES, L. F., VAISBUCH, E., MAZAKI-TOVI, S., HASSAN, S. S. & COSTERTON, J. W. 2008. Detection of a microbial biofilm in intraamniotic infection. *Am J Obstet Gynecol,* 198**,** 135.e1-5.

ROSZAK, D. B., GRIMES, D. J. & COLWELL, R. R. 1984. Viable but nonrecoverable stage of Salmonella enteritidis in aquatic systems. *Can J Microbiol,* 30**,** 334-8.

ROWE, B., BEGG, N. T., HUTCHINSON, D. N., DAWKINS, H. C., GILBERT, R. J., JACOB, M., HALES, B. H., RAE, F. A. & JEPSON, M. 1987. Salmonella ealing infections associated with consumption of infant dried milk. *Lancet,* 2**,** 900-3.

RUSHDY, A. A., STUART, J. M., WARD, L. R., BRUCE, J., THRELFALL, E. J., PUNIA, P. & BAILEY, J. R. 1998. National outbreak of Salmonella senftenberg associated with infant food. *Epidemiology and Infection,* 120**,** 125-128.

RUSSELL, S. M. 2012. *Controlling Salmonella in Poultry Production and Processing*, CRC Press.

RYCHLIK, I. & BARROW, P. A. 2005. *Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection*.

RYSER, E. T. & MARTH, E. H. 2007. *Listeria, Listeriosis, and Food Safety, Third Edition*, Taylor & Francis.

SALIPANTE, S. J., SENGUPTA, D. J., CUMMINGS, L. A., LAND, T. A., HOOGESTRAAT, D. R. & COOKSON, B. T. 2015. Application of Whole-Genome Sequencing for Bacterial Strain Typing in Molecular Epidemiology. *Journal of Clinical Microbiology,* 53**,** 1072-1079.

SCHAECHTER, M. 2009. *Encyclopedia of Microbiology*, Elsevier Science.

SCHATTEN, H. & EISENSTARK, A. 2007. *Salmonella: Methods and Protocols,* New Jersey, Humana Press.

SEIXAS, R., MACHADO, J., BERNARDO, F., VILELA, C. & OLIVEIRA, M. 2014. Biofilm formation by Salmonella enterica serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic, and socio-geographic analysis. *Curr Microbiol,* 68**,** 670-7.

SESHASAYEE, A. S. N., FRASER, G. M., BABU, M. M. & LUSCOMBE, N. M. 2009. Principles of transcriptional regulation and evolution of the metabolic system in E. coli. *Genome Research,* 19**,** 79-91.

SEZONOV, G., JOSELEAU-PETIT, D. & D'ARI, R. 2007. Escherichia coli Physiology in Luria-Bertani Broth. *Journal of Bacteriology,* 189**,** 8746-8749.

SHEN, Y., STOJICIC, S. & HAAPASALO, M. 2011. Antimicrobial Efficacy of Chlorhexidine against Bacteria in Biofilms at Different Stages of Development. *Journal of Endodontics,* 37**,** 657-661.

SHI, X. & ZHU, X. 2009. Biofilm formation and food safety in food industries. *Trends in Food Science & Technology,* 20**,** 407-413.

SHIELDS, P. & CATHCART, L. 2013. *Motility Test Medium Protocol* [Online]. Washington: American Society for Microbiology. Available:

http://www.microbelibrary.org/library/laboratory-test/3658-motility-test-medium-protocol [Accessed 30/01/2015 2015].

SHIVAPRASAD, H. L. 2000. Fowl typhoid and pullorum disease. *Rev Sci Tech,* 19**,** 405-24.

SINDE, E. & CARBALLO, J. 2000. Attachment of Salmonella spp. and Listeria monocytogenes to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology,* 17**,** 439-447.

SMITH, K. & HUNTER, I. S. 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. *Journal of Medical Microbiology,* 57**,** 966-973.

SOMERVILLE, G. A. & PROCTOR, R. A. 2009. At the Crossroads of Bacterial Metabolism and Virulence Factor Synthesis in Staphylococci. *Microbiology and Molecular Biology Reviews : MMBR,* 73**,** 233-248.

SOSINSKY, G. E., FRANCIS, N. R., DEROSIER, D. J., WALL, J. S., SIMON, M. N. & HAINFELD, J. 1992. Mass determination and estimation of subunit stoichiometry of the bacterial hook-basal body flagellar complex of Salmonella typhimurium by scanning transmission electron microscopy. *Proceedings of the National Academy of Sciences,* 89**,** 4801-4805.

SPOERING, A. L. & LEWIS, K. 2001. Biofilms and Planktonic Cells of Pseudomonas aeruginosa Have Similar Resistance to Killing by Antimicrobials. *Journal of Bacteriology,* 183**,** 6746-6751.

SREY, S., JAHID, I. K. & HA, S.-D. 2013. Biofilm formation in food industries: A food safety concern. *Food Control,* 31**,** 572-585.

STEPANOVIC, S., CIRKOVIC, I., RANIN, L. & SVABIC-VLAHOVIC, M. 2004. Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. *Lett Appl Microbiol,* 38**,** 428-32.

STRAWN, L. K. & DANYLUK, M. D. 2010. Fate of Escherichia coli O157:H7 and Salmonella spp. on fresh and frozen cut mangoes and papayas. *International Journal of Food Microbiology,* 138**,** 78-84.

STRINGFELLOW, K., ANDERSON, P., CALDWELL, D., LEE, J., BYRD, J., MCREYNOLDS, J., CAREY, J., NISBET, D. & FARNELL, M. 2009. Evaluation of disinfectants commonly used by the commercial poultry industry under simulated field conditions. *Poultry Science,* 88**,** 1151-1155.

STUMBO, C. R. 2013. *Thermobacteriology in Food Processing*, Elsevier Science.

SWANSON, E. C. & COLLINS, M. T. 1980. Use of the API 20E system to identify veterinary Enterobacteriaceae. *Journal of Clinical Microbiology,* 12**,** 10-14.

TABOADA, E. N., LUEBBERT, C. C. & NASH, J. H. 2007. Studying bacterial genome dynamics using microarray-based comparative genomic hybridization. *Methods Mol Biol,* 396**,** 223-53.

TAKAHASHI, H., KURAMOTO, S., MIYA, S. & KIMURA, B. 2011. Desiccation survival of Listeria monocytogenes and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. *Food Control,* 22**,** 633-637.

TAM, C., RODRIGUES, L., VIVIANI, L., DODDS, J., EVANS, M., HUNTER, P., GRAY, J., LETLEY, L., RAIT, G., TOMPKINS, D. S. & O'BRIEN, S. J. 2012. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. *Gut,* 61**,** 69-77.

TAMMAKRITSADA, M. & TODHANAKASEM, T. 2012. Isolation of Salmonella from Natural Sources Representing High Potential for Biofilm Formations. *Journal of AU Technology,* 15**,** 225-232.

TAN, M. S., WANG, Y. & DYKES, G. A. 2013. Attachment of bacterial pathogens to a bacterial cellulose-derived plant cell wall model: a proof of concept. *Foodborne Pathog Dis,* 10**,** 992-4.

TANG, Y. W., LIU, D., POXTON, I., SCHWARTZMAN, J. & SUSSMAN, M. 2001. *Molecular Medical Microbiology, Three-Volume Set*, Elsevier Science.

TANG, Y. W., SUSSMAN, M., LIU, D., POXTON, I. & SCHWARTZMAN, J. 2014. *Molecular Medical Microbiology, Three-Volume Set*, Elsevier Science.

TITTSLER, R. P. & SANDHOLZER, L. A. 1936. The Use of Semi-solid Agar for the Detection of Bacterial Motility. *Journal of Bacteriology,* 31**,** 575-580.

TO, M. S., FAVRIN, S., ROMANOVA, N. & GRIFFITHS, M. W. 2002. Postadaptational Resistance to Benzalkonium Chloride and Subsequent Physicochemical Modifications of Listeria monocytogenes. *Applied and Environmental Microbiology,* 68**,** 5258-5264.

TODAR, K. 2012. *Salmonella and Salmonellosis,* Wisconsin, Todars Online Textbook of Bacteriology.

TONDO, E. C., MACHADO, T. R. M., MALHEIROS, P. D. S., PADRÃO, D. K., DE CARVALHO, A. L. & BRANDELLI, A. 2010. Adhesion and biocides inactivation of Salmonella on stainless steel and polyethylene. *Brazilian Journal of Microbiology,* 41**,** 1027-1037.

TRACY, B., EDWARDS, K. & EISENSTARK, A. 2002. Carbon and nitrogen substrate utilization by archival Salmonella typhimurium LT2 cells. *BMC Evolutionary Biology,* 2**,** 14.

ULLMANN, A. 2012. *Selected Papers in Molecular Biology by Jacques Monod*, Elsevier Science.

VAN EL, C. G., CORNEL, M. C., BORRY, P., HASTINGS, R. J., FELLMANN, F., HODGSON, S. V., HOWARD, H. C., CAMBON-THOMSEN, A., KNOPPERS, B. M., MEIJERS-HEIJBOER, H., SCHEFFER, H., TRANEBJAERG, L., DONDORP, W. & DE WERT, G. M. W. R. 2013. Whole-genome sequencing in health care. *Eur J Hum Genet,* 21**,** 580-584.

VAN IMMERSEEL, F., DE ZUTTER, L., HOUF, K., PASMANS, F., HAESEBROUCK, F. & DUCATELLE, R. 2009. Strategies to control Salmonella in the broiler production chain. *World's Poultry Science Journal,* 65**,** 367-392.

VELUZ, G. A., PITCHIAH, S. & ALVARADO, C. Z. 2012. Attachment of Salmonella serovars and Listeria monocytogenes to stainless steel and plastic conveyor belts. *Poultry Science,* 91**,** 2004-2010.

VESTBY, L., MORETRO, T., BALLANCE, S., LANGSRUD, S. & NESSE, L. 2009a. Survival potential of wild type cellulose deficient Salmonella from the feed industry. *BMC Vet Res,* 5**,** 43.

VESTBY, L., MORETRO, T., LANGSRUD, S., HEIR, E. & NESSE, L. 2009b. Biofilm forming abilities of Salmonella are correlated with persistence in fish meal- and feed factories. *BMC Veterinary Research,* 5**,** 20.

VU, B., CHEN, M., CRAWFORD, R. & IVANOVA, E. 2009. Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. *Molecules,* 14**,** 2535-2554.

WAIN, J., HOUSE, D., ZAFAR, A., BAKER, S., NAIR, S., KIDGELL, C., BHUTTA, Z., DOUGAN, G. & HASAN, R. 2005. Vi Antigen Expression in Salmonella enterica Serovar Typhi Clinical Isolates from Pakistan. *Journal of Clinical Microbiology,* 43**,** 1158-1165.

WALDNER, L. L., MACKENZIE, K. D., KÖSTER, W. & WHITE, A. P. 2012. From Exit to Entry: Long-term Survival and Transmission of Salmonella. *Pathogens,* 1**,** 128-155.

WALTHERS, D., TRAN, V. K. & KENNEY, L. J. 2003. Interdomain Linkers of Homologous Response Regulators Determine Their Mechanism of Action. *Journal of Bacteriology,* 185**,** 317-324.

WARWICK, U. O. 2010. *Scanning Electron Microscopy (SEM)* [Online]. Available: http://www2.warwick.ac.uk/fac/sci/physics/current/postgraduate/regs/mpags/ex5/techniques/structural/sem3 [Accessed 11/01/2014.

WHEELER, J. G., SETHI, D., COWDEN, J. M., WALL, P. G., RODRIGUES, L. C., TOMPKINS, D. S., HUDSON, M. J. & RODERICK, P. J. 1999. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. *BMJ : British Medical Journal,* 318**,** 1046-1050.

WHO, W. H. O. 2010. Global Salm-Surv -A global Salmonella surveillance and laboratory support project of the World Health Organization. *In:* HENDRIKSEN, R. S. (ed.) *MIC determination by broth dilution using Sensititre.* 4th ed.

WILKS, S. A., MICHELS, H. T. & KEEVIL, C. W. 2006. Survival of Listeria monocytogenes Scott A on metal surfaces: implications for cross-contamination. *Int J Food Microbiol,* 111**,** 93-8.

WONG, H. S., TOWNSEND, K. M., FENWICK, S. G., MAKER, G., TRENGOVE, R. D. & O'HANDLEY, R. M. 2010a. Comparative susceptibility of Salmonella Typhimurium biofilms of different ages to disinfectants. *Biofouling,* 26**,** 859-64.

WONG, H. S., TOWNSEND, K. M., FENWICK, S. G., TRENGOVE, R. D. & O'HANDLEY, R. M. 2010b. Comparative susceptibility of planktonic and 3-day-old Salmonella Typhimurium biofilms to disinfectants. *J Appl Microbiol,* 108**,** 2222-8.

WOODWARD, M. J., GETTINBY, G., BRESLIN, M. F., CORKISH, J. D. & HOUGHTON, S. 2002. The efficacy of Salenvac, a Salmonella enterica subsp. Enterica serotype Enteritidis iron-restricted bacterin vaccine, in laying chickens. *Avian Pathol,* 31**,** 383-92.

WORLD HEALTH ORGANIZATION, W. 2013a. *Salmonella (non-typhoidal)* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs139/en/ [Accessed 30/11/2014 2014].

WORLD HEALTH ORGANIZATION, W. 2013b. *Salmonella (non-typhoidal)* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs139/en/ [Accessed 04/12/2014.

WRAY, C. & WRAY, A. 2000. *Salmonella in Domestic Animals*, CABI Pub.

YANG, S. J., PARK, K. Y., KIM, S. H., NO, K. M., BESSER, T. E., YOO, H. S., KIM, S. H., LEE, B. K. & PARK, Y. H. 2002. Antimicrobial resistance in Salmonella enterica serovars Enteritidis and Typhimurium isolated from animals in Korea: comparison of phenotypic and genotypic resistance characterization. *Veterinary Microbiology,* 86**,** 295-301.

YI, S., XIE, J., LIU, N., LI, P., XU, X., LI, H., SUN, J., WANG, J., LIANG, B., YANG, C., WANG, X., HAO, R., WANG, L., WU, Z., ZHANG, J., WANG, Y., HUANG, L., SUN, Y., KLENA, J. D., MENG, J., QIU, S. & SONG, H. 2014. Emergence and prevalence of non-H2S-producing Salmonella enterica serovar Senftenberg isolates belonging to novel sequence type 1751 in China. *J Clin Microbiol,* 52**,** 2557-65.

YOUNG, K. D. 2007. Bacterial morphology: Why have different shapes? *Current opinion in microbiology,* 10**,** 596-600.

ZWIETERING, M. H., DE WIT, J. C., CUPPERS, H. G. A. M. & VAN 'T RIET, K. 1994. Modeling of Bacterial Growth with Shifts in Temperature. *Applied and Environmental Microbiology,* 60**,** 204-213.

10 Appendix

10.1 Table 29 Summary descriptive statistics on the raw data

RAW DATA	well	Valid N	Mean	Median	Mode	Frequency of mode	Minimum	Maximum	Lower Quartile	Upper Quartile	Quartile Range	Std.Dev.
Negative Control	A1	44	0.392159	0.409000	.3100000	3	0.287000	0.496000	0.311500	0.445000	0.133500	0.068530
D-Raffinose	B1	44	0.576902	0.559000	Multiple	2	0.333290	0.885000	0.476000	0.648500	0.172500	0.152412
a-D-Glucose	C1	44	0.715622	0.640000	Multiple	2	0.520000	1.201000	0.579500	0.802500	0.223000	0.194513
D-Sorbitol	D1	44	0.806688	0.794500	.2240000	3	0.224000	1.226000	0.745500	0.967000	0.221500	0.233167
Gelatin	E1	44	0.578409	0.626000	Multiple	2	0.181000	0.803000	0.518500	0.710000	0.191500	0.174398
Pectin	F1	44	0.541609	0.552000	Multiple	2	0.187000	0.804000	0.473500	0.649000	0.175500	0.163202
p-Hydroxyl- Phenylacetic	G1	44	0.971727	1.032500	.1600000	3	0.160000	1.310000	0.920500	1.204500	0.284000	0.318338
Tween 40	H1	44	0.750318	0.803500	.2070000	3	0.207000	1.304000	0.671500	0.886500	0.215000	0.245505
Dextrin	A2	44	0.912465	0.919000	Multiple	2	0.723000	1.086440	0.862500	0.977500	0.115000	0.086606
a-D-Lactose	B2	44	0.657909	0.530000	.5110000	3	0.414000	1.807000	0.470000	0.665500	0.195500	0.370703
D-Mannose	C2	44	0.866176	0.795500	Multiple	2	0.691000	1.370000	0.758500	0.902500	0.144000	0.183764
D-Mannitol	D2	44	0.844591	0.756000	Multiple	2	0.530000	1.781000	0.669500	0.910500	0.241000	0.307371
Glycyl-L-Proline	E2	44	1.041568	1.148500	Multiple	2	0.270000	1.337000	1.004500	1.209500	0.205000	0.273034
D-Galacturonic Acid	F2	44	0.704227	0.718000	Multiple	2	0.320000	0.938000	0.642000	0.813500	0.171500	0.156504
Methyl Pyruvate	G2	44	0.918955	1.048000	Multiple	2	0.130000	1.262000	0.936000	1.110000	0.174000	0.346731
g-Amino-Butryric Acid	H2	44	0.506682	0.440000	Multiple	2	0.140000	1.308000	0.364000	0.505500	0.141500	0.295704
D-Maltose	A3	44	0.818507	0.785500	Multiple	2	0.608000	1.170000	0.723500	0.900155	0.176655	0.132019
D-Melibiose	B3	44	0.912481	0.940500	.9220000	3	0.483000	1.177900	0.875500	1.008500	0.133000	0.158038
D-Fructose	C3	44	0.881647	0.844000	Multiple	2	0.699000	1.322000	0.776500	0.881500	0.105000	0.167665
D-Arabitol	D3	44	0.752826	0.755000	Multiple	2	0.478000	1.059000	0.665000	0.792500	0.127500	0.137230
L-Alanine	E3	44	0.981866	1.031000	.4430000	3	0.443000	1.205000	0.927000	1.116000	0.189000	0.192237
L-Galactonic Acid Lactone	F3	44	0.819886	0.825500	Multiple	2	0.310000	1.496000	0.653000	0.953500	0.300500	0.283308
D-lactoc acid Methyl Ester	G3	44	0.896323	0.907000	Multiple	2	0.244200	1.687000	0.754500	1.016000	0.261500	0.319287
a-Hydroxy-Butyric Acid	H3	44	0.756477	0.814000	Multiple	2	0.181000	1.072000	0.677500	0.904500	0.227000	0.212585
D-Trehalose	A4	44	0.794623	0.757000	1.025000	3	0.552000	1.102491	0.721500	0.853000	0.131500	0.148038
b-Methyl-DGlucoside	B4	44	0.807130	0.803500	1.064000	3	0.552000	1.308000	0.646150	0.868000	0.221850	0.205932
D-Galactose	C4	44	0.977513	0.984000	Multiple	2	0.467593	1.393000	0.926000	1.053500	0.127500	0.207006
myo-Inositol	D4	44	0.830659	0.806500	Multiple	2	0.604000	1.323000	0.744500	0.856500	0.112000	0.169651
L-Arginine	E4	44	0.867737	0.903000	Multiple	2	0.440334	1.053000	0.809500	0.957500	0.148000	0.145460
D-Gluconic Acid	F4	44	1.209489	1.297500	.4430000	3	0.437534	1.513000	1.212500	1.341500	0.129000	0.273841
L-Lactic Acid	G4	44	1.166732	1.257000	Multiple	2	0.381000	1.401000	1.163000	1.302500	0.139500	0.267813
β-Hydroxy-D,Lbutyric acid	H4	44	0.488122	0.430500	Multiple	2	0.301000	1.228000	0.365500	0.494000	0.128500	0.226706
D-Cellobiose	A5	44	0.695227	0.598000	Multiple	2	0.414000	1.645000	0.520000	0.659000	0.139000	0.320354
D-Salicin	B5	44	0.823623	0.710700	Multiple	2	0.413000	2.203000	0.583500	0.806500	0.223000	0.423157
3-Methyl Glucose	C5	44	0.798043	0.770500	Multiple	2	0.476000	1.754000	0.631000	0.798500	0.167500	0.313033
Glycerol	D5	44	1.176955	1.241000	1.009000	2	0.737000	1.408000	1.056500	1.307000	0.250500	0.173819
L-Aspartic Acid	E5	44	1.238333	1.305500	Multiple	2	0.476666	1.460000	1.237000	1.372000	0.135000	0.255827
D-Glucuronic Acid	F5	44	1.245089	1.335000	.5580000	3	0.558000	1.499000	1.208000	1.392000	0.184000	0.243970
Citric Acid	G5	44	1.216861	1.265000	.5380000	3	0.530000	1.700000	1.185950	1.366000	0.180050	0.250429
a-Keto-Butyric Acid	H5	44	0.753705	0.742000	.4120000	3	0.412000	1.013000	0.655000	0.885500	0.230500	0.157980
Gentiobiose	A6	44	0.689295	0.645000	1.071000	3	0.359000	1.467000	0.516500	0.728000	0.211500	0.276136
N-Acetyl-DGlucosamine	B6	44	0.859394	0.773000	Multiple	2	0.579000	1.574000	0.712500	0.926500	0.214000	0.239603
D-Fucose	C6	44	0.859705	0.866000	.8660000	3	0.510000	1.528000	0.760500	0.886000	0.125500	0.219455
D-Glucose- 6-PO4	D6	44	1.295321	1.394500	.4960000	3	0.484121	1.526000	1.316500	1.444000	0.127500	0.281743
L-Glutamic Acid	E6	44	1.117295	1.137500	.4690000	3	0.469000	1.881000	1.018000	1.216500	0.198500	0.272053
Glucuronamide	F6	44	1.100704	1.085500	Multiple	2	0.577000	1.827000	0.993500	1.144500	0.151000	0.277699
a-Keto-Glutaric Acid	G6	44	0.609114	0.589500	.5170000	3	0.413000	0.915000	0.517000	0.679500	0.162500	0.122548
Acetoacetic Acid	H6	44	0.688683	0.679500	Multiple	2	0.474000	0.855000	0.601000	0.798021	0.197021	0.117342

RAW DATA	well	Valid N	Mean	Median	Mode	Frequency of mode	Minimum	Maximum	Lower Quartile	Upper Quartile	Quartile Range	Std.Dev.
Sucrose	A7	44	0.489160	0.477000	.4230000	3	0.320000	0.636000	0.423000	0.566500	0.143500	0.084162
N-Acetyl-b-DMannosamine	B7	44	1.050568	1.079000	1.097000	3	0.715000	1.304000	0.918500	1.163000	0.244500	0.157697
L-Fucose	C7	44	1.008295	1.033000	.5610000	3	0.559000	1.310000	0.959500	1.083000	0.123500	0.176432
D- Fructose 6-PO4	D7	44	1.280250	1.347500	Multiple	2	0.584000	1.505000	1.314500	1.412500	0.098000	0.248291
L-Histidine	E7	44	1.009300	1.039500	.5680000	3	0.568000	1.172000	0.992500	1.109000	0.116500	0.156353
Mucic Acid	F7	44	1.191205	1.261000	1.255000	3	0.500000	1.387000	1.144500	1.328500	0.184000	0.242526
D-Malic Acid	G7	44	0.512136	0.519500	.3630000	3	0.363000	0.715000	0.446500	0.568000	0.121500	0.098332
Propionic Acid	H7	44	0.926045	0.940500	.6850000	3	0.553000	1.218000	0.844500	1.043000	0.198500	0.158955
D-Turanose	A8	44	0.662711	0.625500	Multiple	2	0.384000	1.452000	0.513000	0.678500	0.165500	0.247264
N-Acetyl-DGalactosamine	B8	44	0.679161	0.673000	.6400000	4	0.460000	0.935000	0.566500	0.763350	0.196850	0.129360
L-Rhamnose	C8	44	1.096636	1.045500	Multiple	2	0.890000	1.749000	0.946000	1.135500	0.189500	0.227270
D-Aspartic Acid	D8	44	1.197795	1.278000	Multiple	2	0.483000	1.476000	1.183000	1.331500	0.148500	0.256570
L-Pyroglutamic Acid	E8	44	0.839364	0.838000	Multiple	2	0.502000	1.110000	0.758000	0.965000	0.207000	0.150936
Quinic Acid	F8	44	0.769165	0.782500	Multiple	2	0.489000	1.014000	0.692500	0.864000	0.171500	0.142556
L-Malic Acid	G8	44	1.199341	1.233000	.5240000	3	0.524000	1.990000	1.151000	1.323500	0.172500	0.272838
Acetic Acid	H8	44	0.977488	1.014500	.6690000	2	0.644000	1.284000	0.897000	1.087000	0.190000	0.176245
Stachyose	A9	44	0.570043	0.569500	Multiple	2	0.325000	0.772000	0.495000	0.653000	0.158000	0.115637
N- Acetyl Neuraminic Acid	B9	44	0.977345	1.023000	Multiple	2	0.471000	1.219000	0.935500	1.088000	0.152500	0.193486
inosine	C9	44	1.317359	1.378500	Multiple	2	0.564795	1.645000	1.309000	1.474000	0.165000	0.272616
D-Serine	D9	44	1.174250	1.344000	.4850000	2	0.372000	1.503000	1.124000	1.401000	0.277000	0.365750
L-Serine	E9	44	1.222027	1.289500	Multiple	2	0.501200	1.452000	1.184500	1.378500	0.194000	0.251577
D-Saccharic Acid	F9	44	1.228065	1.308000	.5690000	2	0.569000	1.484000	1.173000	1.386500	0.213500	0.242468
Bromo-Succinic Acid	G9	44	1.089440	1.166000	Multiple	3	0.484000	1.420000	0.989500	1.233500	0.244000	0.248281
Formic Acid	H9	44	0.556391	0.552000	.5520000	3	0.377000	0.775000	0.472000	0.629500	0.157500	0.118918
Positive Control	A10	44	1.647000	1.680000	Multiple	2	1.020000	1.893000	1.615500	1.759000	0.143500	0.207534
1% NaCl	B10	44	1.691250	1.737000	Multiple	2	1.023000	1.907000	1.630500	1.854000	0.223500	0.227474
1% sodium Lactate	C10	44	1.733609	1.746500	1.044000	3	1.020000	2.203000	1.685500	1.874000	0.188500	0.257815
Troleandomycin	D10	44	1.576386	1.652500	Multiple	2	0.465989	2.054000	1.575000	1.711000	0.136000	0.371248
Lincomycin	E10	44	1.531480	1.621500	Multiple	2	0.488100	1.827000	1.526000	1.711000	0.185000	0.341652
Vancomycin	F10	44	1.708250	1.815500	.5400000	3	0.510000	2.100000	1.734500	1.892500	0.158000	0.390354
Nalidixic Acid	G10	44	0.727386	0.521000	Multiple	2	0.176000	1.748000	0.373000	1.093000	0.720000	0.462184
Aztreonam	H10	44	0.703205	0.729000	1.074000	3	0.246000	1.099000	0.442500	0.908000	0.465500	0.257517
pH 6	A11	44	1.662136	1.646500	1.140000	3	1.130000	1.926000	1.598500	1.825000	0.226500	0.201459
4% NaCl	B11	44	1.394454	1.405500	Multiple	2	0.955000	1.687000	1.329000	1.519000	0.190000	0.182032
Fusidic Acid	C11	44	1.444911	1.503500	1.585000	3	0.507000	1.754000	1.416000	1.613500	0.197500	0.317922
Rifamycin SV	D11	44	1.735824	1.808500	Multiple	2	0.482246	2.164000	1.724000	1.974000	0.250000	0.422139
Guanidine HCl	E11	44	1.470630	1.478500	Multiple	2	0.964000	1.742000	1.382000	1.639000	0.257000	0.195881
Tetrazolium violet	F11	44	3.687666	3.963000	Multiple	2	0.969288	4.106000	3.887000	4.008500	0.121500	0.857228
Lithium Chloride	G11	44	1.196527	1.157000	Multiple	2	1.004000	1.497000	1.082500	1.322000	0.239500	0.144205
pH 5	A12	44	1.418745	1.453500	1.460000	3	0.830000	1.781000	1.366500	1.510000	0.143500	0.212680
8% NaCl	B12	44	0.833145	0.809500	Multiple	2	0.528000	1.222000	0.720000	0.905500	0.185500	0.184421
D-Serine	C12	44	1.090534	1.146000	1.128000	3	0.296000	1.485000	0.990000	1.252500	0.262500	0.295754
Minocycline	D12	44	0.614140	0.446000	Multiple	2	0.210000	1.498000	0.386500	0.679000	0.292500	0.378432
Niaproof 4	E12	44	1.590500	1.613000	Multiple	2	0.600000	2.253000	1.456000	1.843000	0.387000	0.380668
Tetrazolium Blue	F12	44	3.307864	3.578500	.7060000	3	0.706000	3.825000	3.446000	3.689500	0.243500	0.849888
Potassium Tellurite	G12	44	0.741568	0.856500	Multiple	2	0.180000	1.320000	0.345000	1.068000	0.723000	0.396762
Sodium Bromate	H12	44	0.661053	0.650000	Multiple	2	0.514000	0.987000	0.580000	0.716000	0.136000	0.104758

Table 10.1 shows descriptive statistics for absorbance values in each well, the mean, mode, median, upper& lower quartiles ranges are all highlighted. Some wells showed large variability in the range of absorbance values in comparison to others.

10.2 Conferences attended and other professional activities

Poster presentations

Bashir, A., Hilton, A. C (2015) Are there *really Salmonella* superbugs in food factories? University-wide Poster and Three Minute Thesis (3MT) competition, 22nd May, Aston University, Birmingham, UK. **(First Prize)**

Amreen Bashir, Yvonne Stedman and Anthony C. Hilton (2014) Exploring Temperature Resistance and Biofilm Formation as the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments, Postgraduate Poster Day, 2nd July, Aston University, Birmingham, UK.

Amreen Bashir, Yvonne Stedman and Anthony C. Hilton (2013) Exploring the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments, V International Conference on Environmental, Industrial and Applied Microbiology, 2nd - 4th October, Madrid, Spain

Bashir, A., Hilton, A. C., Stedman, Y (2013) Exploring the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments, Early career researchers in food event, Biosciences KTN, London , UK

Bashir, A., Hilton, A. C., Stedman, Y (2013) Exploring the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments, Postgraduate Poster Day, 26th June, Aston University, Birmingham, UK.

Bashir, A., Hilton, A. C., Stedman, Y (2012) Exploring the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments, Postgraduate Poster Day, 27th June, Aston University, Birmingham, UK.

Bashir, A., Hilton, A. C (2012) Are there *really Salmonella* superbugs in food factories? University wide Poster competition, 3rd July, Aston University, Birmingham, UK (runner up)

Oral Presentations

Bashir, A (2015) Exploring the biological basis for *Salmonella* Persistence in Food Manufacturing Environments, Research in Progress Seminar (RIP), 21st January, Aston University, Birmingham, UK.

Bashir, A (2014) *Salmonella* PhD project update, 9th June, Mars, Slough, UK

Bashir, A (2014) Exploring the biological basis for *Salmonella* Persistence in Food Manufacturing Environments, Research in Progress Seminar (RIP), 9th February, Aston University, Birmingham, UK.

Bashir, A (2014) Are there *really Salmonella* superbugs in food Manufacturing environments factories? University-wide Poster and Three Minute Thesis (3MT) competition, 22nd May, Aston University, Birmingham, UK.

Bashir, A (2012) Control of *Salmonella* in food environments, Research in Progress Seminar (RIP), 18th June, Aston University, Birmingham, UK.

Professional qualifications obtained

2012-2014 Postgraduate Certificate in Professional Practice in Higher Education (PGCPP) Aston University, Birmingham, UK

2011-2012 Postgraduate Certificate of teaching and learning in Higher Education (PGcert) 22nd May, Aston University, Birmingham, UK

Science communication and public engagement

ECCSMID conference (2014) 10th to 13th May, 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain

Microbiology Road Show (2011 – 2014) Participation in 'Microbiology in Schools' scheme, funded by the Welcome Trust, Aston University, Birmingham, UK

Society of Applied Microbiology (SFAM) (2011-2013), Disease detective at Big Bang Fair, NEC, Birmingham, UK

Society of Applied Microbiology (SFAM) (2013), Summer conference, 1st - 4th July, Cardiff, UK

Society of Applied Microbiology (SFAM) (2013), STI's in the 21st century' Spring meeting, 24th April, Stratford Upon Avon, UK

Assistant tutor and Demonstrator for Microbiology undergraduate modules including Clinical and Food microbiology & Biotechnology. 2011-2014.

Aston University

This is to certify that

Amreen Bashir

won 1st prize in the

Postgraduate Researchers
Poster Competition

22 May 2015

Peter Lakeland
HR - Staff & Graduate Development Manager

Are there *really Salmonella* superbugs in food factories?

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Amreen Bashir, Professor Anthony Hilton & Dr Yvonne Stedman

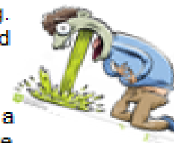
Background to project

- Food factories invest time and money to ensure outbreaks linked to contaminated food are kept to a minimum. However despite strategies to prevent the spread of pathogens like *Salmonella* during food manufacture, notable outbreaks still occur
- In **2008** reports of *Salmonella* were associated with dry dog food from a leading manufacturer.
- It caused over 70 cases of **human** food poisoning. 39% were 1 year old or younger and 32% experienced bloody diarrhoea.
- This is the scenario worldwide as *Salmonella* are a leading cause of bacterial diarrhoea; they are estimated to cause 94 million cases of gastroenteritis and 115,000 deaths globally each year.
- Epidemiological analysis suggests that in some manufacturing processes contamination of the product may have arisen due to environmentally **persistent** strains.

Aims & Objectives

The overall aim was to investigate **how *Salmonella* persists** on food contact and processing surfaces. In this specific study the objectives were:

- To identify whether *Salmonella* were capable of forming biofilms in a 96 well micro titre well plate format
- To investigate if the **food factory** isolates could form stronger biofilms compared to the isolates from other environments



Selecting a panel of isolates

- *Salmonella* isolates representative of those from food, clinical and veterinary environments was created from commercially available culture collections and research archive strains.

What are biofilms?

- These are communities of bacteria that attach to surfaces.
- Studies have suggested enhanced biofilm formation may enable *Salmonella* to persist in the environment by protecting against environmental stress & disinfection.

Experiment and results

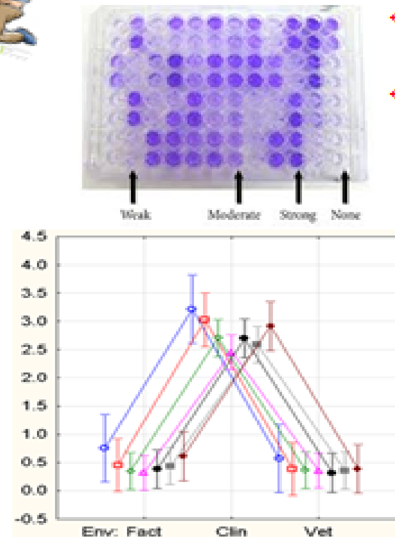


Figure 1 shows an overall summary of the effect of environment, temperature and media on biofilm production

- Conducted a colorimetric assessment of biofilm density at 37°C, 25°C,15°C &10°C in nutritious bacterial growth medium and nutrient deprived media.
- As the plate on the left shows **crystal violet** density was observed in a 96 well microtitre plate. The O.D was measured at 570nm using a spectrophotometer.

Key findings & Conclusion

- All the strains investigated in the panel were able to form biofilms
- None of the factory isolates showed any significantly enhanced ability to form stronger biofilms.
- In all conditions tested only the clinical isolate of S. Senftenberg 775W was statistically significantly better at forming a biofilm.
- Biofilm formation is **unlikely** to **explain** *Salmonella* **persistence** in the factory environment.
- Other investigations have explored parameters such as the characterization of genotype, phenotype and biocide resistance

``The persistence *Salmonella* in food manufacturing environments''

Are they ***really*** superbugs?

By Amreen Bashir
Supervised by Professor Anthony Hilton

3minute thesis script

I am sure everyone in this room has had an experience of food poisoning at some point in their lives. And the excruciating stomach cramps, diarrhoea and fever are not symptoms to easily forget. In the past few decades there have been various outbreaks of salmonella contamination associated with a range of food products such as eggs, pet foods, raw products and cereals. In the UK alone the HPA reports thousands of cases of infections each year. Now the first outbreak that I can actually recall was in 2009 when Cadbury's ended up recalling thousands of bars of chocolates when 40 people were hospitalised – when you're a chocaholic like and your favourites are wiped off the shelf believe me its not a good feeling. Subsequent investigations showed the outbreak was linked to salmonella contamination in the factory and Cadbury's was fined a million pound's plus a 100000 in court costs as well as a product backlash. The fact is these outbreaks can occur despite there being strategies already implemented to prevent the spread of pathogens during the manufacturing stage. Investigations have suggested this may be due to cross contamination of the product with *Salmonella* strains resident within the factory environment. There are over 2500 serotypes of *salmonella* however data has shown the strains causing the outbreaks relating to factories are not the serotypes which are commonly known to cause illness. Therefore the aim of my study was to find out what enables these serotypes to persist in factory environments? and are they ***really*** superbugs? Initially a panel of strains from three environments were selected which consisted of isolates taken from the factory, clinical isolates from patients in hospital and veterinary isolates. These were serotype matched where possible, the reason for this was if there was a potential difference then we wanted to make sure that it was because the factory strains actually possessed something which made them stand out. Next experiments were conducted to find out what what enabling these strains to survive, so we looked at possible ways to almost mimic the factory conditions in the laboratory. We looked at how these strains grow in nutrious media and media with limited nutrients. as well as their ability to survive on steel at different temperatures as many surfaces in factories are made of steel. Other experiments included phenotypically typing the strains this meant looking at how they metabolically different are they using up some sugars more than others. We also looked at their ability to attach to surfaces via biofilm formation and I am currently investigating the effect of a range of disinfectants that can be used to kill these strains. Now although it would have been nice to have found that these strains do actually have something spectacular about them, this has not been the case, my results indicate in all the experiments the factory strains have not outcompeted the other isolates. They are able to grow and survive on surfaces but not better than the other strains. So maybe more attention needs to be paid to complying with cleaning protocols and repairing old parts of buildings where salmonella may be surviving in the debris and maybe coating them. Which overall really isn't a bad result- I mean would you really want there to be a super resistant strain of salmonella in the factories where food products are made.

Exploring the Biological Basis for *Salmonella* Persistence in Food Manufacturing Environments

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Professor Anthony Hilton

Introduction

Despite strategies to prevent the spread of *Salmonella* during food manufacture, notable outbreaks still occur. The sources and routes by which *Salmonella* can contaminate food are numerous, including on raw materials and from the factory environment and personnel. Epidemiological analysis suggests that in some manufacturing processes contamination of the product may have arisen as a consequence of environmentally persistent strains. If this were to be the case, the identification of the underlying mechanisms by which these *Salmonella* establish and survive in the food manufacturing environments is key for the implementation of effective control strategies.

Aims & Objectives

The aim of this study was to investigate how *Salmonella* persists on food contact and processing surfaces. Specific objectives were:

- To create a panel of test isolates in the categories of clinical, veterinary and factory isolates
- To design a standard method to test survival on steel
- To observe how temperature affects survival of the three categories of isolates on steel
- To identify whether the strains were capable of forming biofilm at 24 hours in a 96 well microtitre well plate format and whether biofilm formation was enhanced at 48 hours
- To investigate the effect of temperature on biofilm formation at 37°C, 25°C, 15°C and 10°C
- To investigate the effect of media/nutrients on biofilm formation using full strength TSB media and 1/20 diluted TSB media

Methods (i)

Salmonella isolates representative of those from food, clinical and veterinary environments were obtained from commercially available culture collections and research archive strains.
These consisted of factory isolates of *S.* Senftenburg, *S.* Schwarzengrund, *S.* Livingstone, *S.* Kedougou, *S.* Montevideo; clinical and veterinary isolates of *S.* Senftenburg, *S.* Schwarzengrund, as well as *S.* Typhimurium SL1344 and *L. monocytogenes* (NCTC11994).

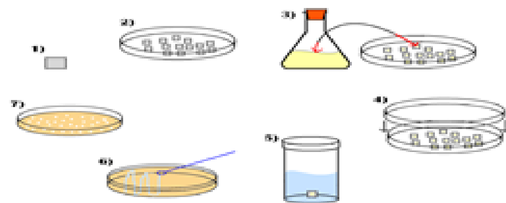

Figure 1: Schematic of survival on steel experiment

To determine the ability of *Salmonella* isolates to survive on surfaces. (1) Steel discs were cut to size (n=30, 1cm²) using a guillotine and autoclaved. (2) Following sterilization they were placed in a sterile Petri dish. (3) The discs were then inoculated with 10µl of an overnight culture of test organism (of approximately 10^8 cfu/ml) and dried. (4) The coupons were incubated at 10°, 15°, 25° & 37°C. (5) At regular time points the discs were immersed in 10ml saline and mixed by vortexing to recover surviving cells. (6) Viable counts of the recovered cells were determined by culture on Nutrient Agar. Decimal Reduction Times (DRT day-1) were calculated for each strain / temperature combination.

Methods (ii)

The ability of the strains to form biofilm was also explored. Enhanced biofilm formation may enable *Salmonella* to persist in the environment by protecting against environmental stress. A colorimetric assessment of biofilm density at 37°C, 25°C, 15°C & 10°C in TSB media and 1/20 TSB media using crystal violet was observed in a 96 well microtitre plate. The O.D was measured at 570nm using a Bio-Tek incubating spectrophotometer. The level of biofilm formation was assessed at 24 hours and again at 48 hours following a media change at 24 hours. Factorial ANOVA analysis was used to highlight the main factors influencing biofilm forming ability of the strains.

Survival Results (i)

Strain	10°C Steel	15°C Steel	25°C Steel	37°C Steel
S.Typhimurium SL1344	7.0	21.1	40.3	0.01325
Listeria monocytogenes NCTC11994	3.0	9.38	30.0	0.00834
S.Senftenburg Clinical 775W	5.9	15.5	15.8	0.14
S.Senftenburg Vet	5.47	22.7	33.1	0.13
S.Senftenburg FSO	6.39	25.6	34.4	0.13
S.Schwarzengrund Clinical FSL 95-436	10.0	20.3	36.4	0.13
S.Schwarzengrund Vet	6.30	24.1	40.0	0.0329
S.Schwarzengrund USS	7.36	24.5	36.3	0.13
S.Montevideo	7.62	30.0	30.5	0.0363
S.Livingstone	7.7	23.5	43.46	0.1323
S.Kedougou	4.9	14.6	42.0	0.136

Table 2: Survival of *Salmonella* on stainless steel expressed as DRT (days)

- The mean decimal reduction time (DRT) in days was calculated from 3 observations of the experiment in duplicate. At 10°C the highest D value was for the clinical strain of *Schwarzengrund* D10=10. The average D value of the factory strains was between 6-7 days and the remaining strains revealed D values ranging between 5 and 7 days. The lowest D value was for *L.monocytogenes* D=3.
- At 15°C the factory strain of *S.*Montevideo had the highest D value, D15=30. The remainder of D values ranged from 21-25 days. With the exception of the factory strain of *S.*Kedougou which demonstrated a D15=14.6, the clinical strain of *S.*Senftenburg demonstrated a D value of D15=15.5 and *L.monocytogenes* which showed a D15=9.38
- At 25°C the D value of *S.*Typhimurium (SL1344) was 40 days, the D value of *S.*Livingstone was 43 days and the remainder of the strains returned an average D25=30-42 days. Similarly to 15°C the lowest D values were observed for *S.*Senftenburg 775w D25=15.8 and the veterinary strain of *S.*Senftenburg D25=33.1. At 37°C survival was observed after 48hours for all strains tested.
- Therefore preliminary analysis does not reveal any significantly enhanced survival of factory isolates of *Salmonella* at 10°C, 15°C, 25°C and 37°C compared to matched strains.

Biofilm Results (ii)

Effect of Temperature on biofilm formation

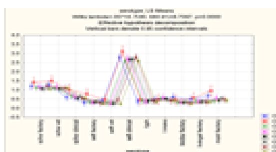

Figure 2: Serotype*Time at 37°C

Results at 37°C show that all the strains can form biofilms and *S.* Senftenburg clinical formed the highest level of biofilm, followed by the factory strain of *S.* Schwarzengrund the veterinary strain of *S.* Schwarzengrund vet. Vertical bars show 95% confidence intervals.

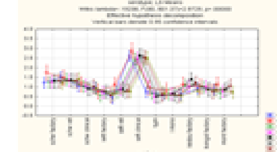

Figure 3: Biofilm formation at 25°C

Results show that all the strains at 25°C were able to form biofilms with *S.* Senftenburg clinic forming the highest level of biofilm followed by the factory strain of *S.* Schwarzengrund. Vertical bars here indicate 95% confidence intervals.

- The same pattern was observed for strains at both 10°C and 15°C.

Effect of Media (TSB and 1/20 TSB) on biofilm formation

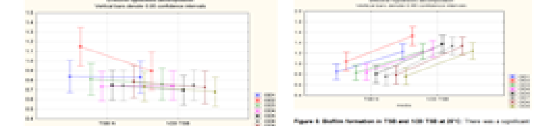

Figure 4: Biofilm formation in TSB and 1/20 TSB at 37°C: Based on 3 observations, results indicate there is no significant difference in biofilm formation in TSB and diluted 1/20 TSB. Vertical bars denote 95% confidence intervals.

Figure 5: Biofilm formation in TSB and 1/20 TSB at 25°C: There was a significant difference in biofilm formation in TSB and 1/20 TSB, with biofilm formation being higher in the diluted media.

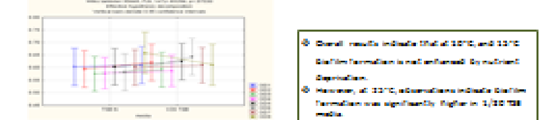

Figure 6: The effect of media effect on biofilm formation at 10°C: Results indicate no significant difference in biofilm formation between TSB and 1/20 TSB media.

- Overall results indicate that at 10°C and 15°C biofilm formation is not enhanced by nutrient deprivation.
- However, at 25°C, observations indicate biofilm formation was significantly higher in 1/20 TSB media.

Effect of Time and Environment on biofilm formation

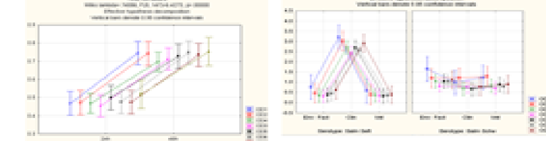

Figure 7: Effect of Time on Biofilm Production at 10°C:

Biofilm production was significantly higher at 48hours compared to 24hours. Vertical bars here indicate 95% confidence intervals

Figure 8: Effect of environment on biofilm formation

The factory strains of *S.* schwarzengrund and *S.* Senftenberg were serotype matched with clinical and veterinary strains. Overall results indicate that only the clinical strain of *S.* Senftenberg was a significantly better biofilm former. Neither of the factory strains showed any significant ability to form stronger biofilms.

- At 37°C, 25°C and 10°C biofilm formation was greater at 48hours compared to 24 hours however this did not achieve significance.

Discussion/Conclusion

- This study suggests that *Salmonella* survival on stainless steel is affected by temperature, atypically with higher survival rates at higher temperatures, however similar findings have been reported previously (1). Resident factories strains did not survive better on stainless steel compared to clinical and veterinary isolates of the same serotype indicating these strains did not demonstrate any temperature adaptation.
- All the strains investigated in the panel were able to form biofilms, other studies have also shown *Salmonella* strains to be good biofilm formers (1,2).
- *Salmonella* survival was better at 30°C than 15°C, in line with published data (1). The current study also showed that biofilm formation was highest at 37°C and decreased at 25°C and 15°C respectively with the lowest level of biofilm formation at 10°C.
- Irrespective of time, media and temperature, the clinical strain of *S.* Senftenburg was the strongest biofilm former. The only other strain which was a strong biofilm former was the factory strain of *S.* Schwarzengrund. Enhanced biofilm formation was not observed in the other factory strains tested.
- In conclusion, environmental survival and biofilm formation are unlikely to explain *Salmonella* persistence in the factory environment. Further investigation is ongoing to explore other parameters which may contribute to persistence including characterization of genotype, phenotype and biocide resistance.

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By Amreen Bashir, Professor Anthony Hilton & Dr Yvonne Stedman
Life and Health Sciences, Aston University, Birmingham, B4 7ET

ABSTRACT

There have been various outbreaks of Salmonella despite strategies to prevent their spread during food manufacture. This may be due to contamination of the product with resident strains, therefore identifying the underlying mechanisms by which *Salmonella* establishes and survives in food factories is key for the implementation of effective control strategies.

This study investigated the survival of factory isolates of *Salmonella* on steel surfaces stored at 10°, 15°, 25° & 37°C, compared to serotype-matched clinical and veterinary strains and L. monocytogenes. Steel discs (n=30; 1cm²) were inoculated with 10µl of an overnight culture of test organism and dried. At regular time points discs were immersed in 10ml SDW and vortexed to recover. Viable counts of the recovered cells were determined by culture on Nutrient Agar.

Decimal Reduction Times were calculated for each strain / temperature combination. At 10°C the D value of the factory S. Schwarzengrund was highest at 3.2 days. Whereas the remainder of the strains had a D value of between 1 and 2.5 days. At 15°C the highest D value was for the veterinary strain of S. Schwarzengrund at 9 days on steel. A Phenotypic microarray was performed to investigate if factory strains of Salmonella demonstrate an enhanced or differential metabolic profile which may contribute towards persistence. Preliminary visual comparison did not reveal any major metabolic differences however more detailed comparison is proposed.

In conclusion, these preliminary data suggests environmental survival is not a major factor influencing persistence of factory strains, and that the surface does not affect persistence, survival studies have been completed at 25°C and 37°C and are pending analysis. Further work is planned to investigate other candidate mechanisms for environmental persistence including biofilm formation and VNC state.

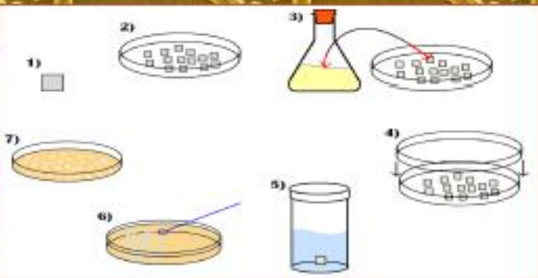


Figure 1: Schematic of steel experiment

(1) Steel discs were cut to size (10mm²) using a guillotine and autoclaved. (2) Following sterilization they were placed in a sterile petri dish. (3)The discs were then inoculated with 10µl of approximately 10^8 Cfu of an overnight culture. (4) The discs were placed in an incubator to dry for 60 minutes at 37°C. (5) at each time point a disc was removed and cultured to determine surviving bacteria, 100 µl of this solution was then plated onto nutrient agar plates (6). Following 18 hour incubation viable counts were recorded in pre-labelled table.

RESULTS

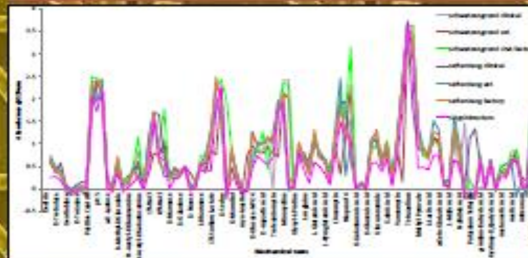
Strain	10°C Steel	10°C Glass	15°C Steel	15°C Glass
S.Typhimurium SL1344	2.5	1.6	5	7.5
Listeria monocytogenes NCTC 11994	1	1.6	4	4
S.Seftenburg Clinical 775w	1.95	1	5.5	3.5
S.Seftenburg Vet	1.27	1.31	5	7.5
S.Seftenburg PBO	1.64	1.69	5	5.5
S.Schwarzengrund Clinical FSL S5-458	1.84	1.65	5.5	8.5
S.Schwarzengrund Vet	1.52	1.22	9	6
S.Schwarzengrund USA	3.2	3.2	3.5	3.5

Table 1: Survival of *Salmonella* on stainless steel & glass

The mean decimal reduction time (DRT) in days was calculated from 3 observations of the experiment in duplicate.
Preliminary analysis does not reveal any significantly enhanced survival of factory isolates of *Salmonella* at 10°C and 15°C, furthermore, the substrate (glass or stainless steel) does not influence survival.

PHENOTYPIC MICROARRAY

- This was performed using biolog plates. A standardised micromethod using 94 biochemical tests. In the plate there are 71 carbon source utilization assays and 23 chemical sensitivity assays
- Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals.
- The absorbance of the 96 well plate was read and recorded, below are images from the test. The deeper purple indicates utilisation of the carbon sources. These results were then displayed as a graph.
- From the graph below, visual comparison reveals general concordance in metabolic profile, which suggests that there is potentially no major metabolic difference between the strains, however the phenotypic microarray will be repeated.



VNC WORK

The Viable non culturable (VNC) state is a recognised phenomenon in *Salmonella*. This experiment explored the question: Does entry into, and subsequent recovery from the VNC state lead to persistence of *Salmonella* in the factory environment?

METHOD

Fluorescence microscopy of strains on stainless steel coupons using the LIVE/DEAD® *BacLight*™ Bacterial Viability Kit. This employs two nucleic acid stains—green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain.

These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO® 9 stain labels both live and dead bacteria.In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red.

S.Schwarzengrund day 1 on steel coupon

S.Schwarzengrund Time 24h on steel 1.87×10^3 cfu/ml

Preliminary data indicates that at 37° strains are not entering the VNC state. The image on the left is predominantly green and shows that the cells are alive whereas the image on the left shows more red and a few green cells so the cells are largely dead with a few live cells, however investigations are ongoing for 25°, 15° and 10° degrees.

CONCLUSION

- The persistence of *Salmonella* on different surfaces is of great concern to the food industry, preliminary visual comparison did not reveal any major metabolic differences however more detailed comparison is proposed.
- On the basis of parameters looked at so far, none are affecting survival and resident factories strains do not seem to be better survivors on stainless steel compared to clinical and veterinary isolates of the same serotype.
- Further investigation is ongoing to looking at other parameters of *Salmonella* biology which may contribute to *Salmonella* persistence such as genotype and biocide resistance.

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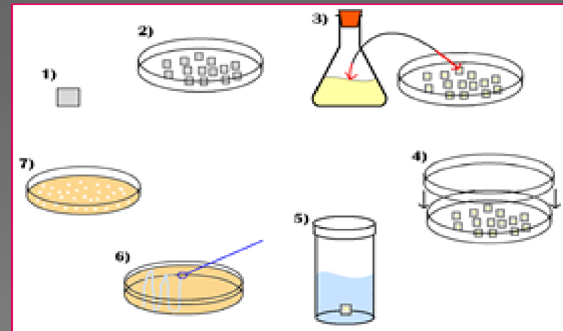
This study investigated the survival of factory isolates of Salmonella on steel surfaces stored at 10°, 15°, 25° & 37°C, compared to serotype-matched clinical and veterinary strains and L monocytogenes. Steel discs (n=30; 1cm^2) were inoculated with 10µl of an overnight culture of test organism and dried. At regular time points discs were immersed in 10ml SDW and vortexed to recover. Viable counts of the recovered cells were determined by culture on Nutrient Agar.

Decimal Reduction Times were calculated for each strain / temperature combination. On steel L monocytogenes had the highest D values at both 10°C (3744 minutes) and a 15°C (19440 minutes). At 10°C the D value of the factory S. Schwarzengrund was 3081minutes and the remaining strains D values of 2100-2800 minutes. At 15°C the D value of the veterinary S. Schwarzengrund was highest at 12960 minutes, whereas the remainder of strains revealed an average D = 7200minutes.

In conclusion, these preliminary data suggests environmental survival is not a major factor influencing persistence of factory strains, however survival studies are ongoing at 25°C and 37°C. Further work is planned to investigate other candidate mechanisms for environmental persistence including biofilm formation and VNC state.



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RESULTS

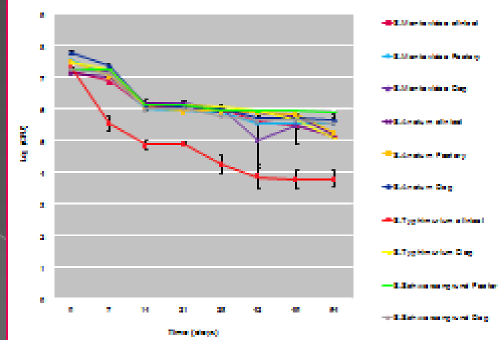

Figure 2: Survival of Salmonella on stainless steel

From a starting Inoculum of between 10^7 and 10^8 at day 0 a majority of the strains regardless of serotype and origin demonstrated a 1 log reduction in population number over the first fourteen days. The clinical isolate of S.Typhimurium being the exception and declining by 2 logs. From day 7 to the end of the experiment at day 54 all of the isolates settled around a log 6 surviving number, representing a 1.5 – 2 log reduction over 54 days at 11degrees C. The notable exception was the clinical isolate of S. Typhimurium which continued declined to a log of 4. The error bars show the standard deviation. In general the error bars where small indicating that the data was close to the mean. The notable exceptions beings the clinical strain of S.Typhimurium and the veterinary strain of S.Montevideo at 42 days which both had large error bars.

SUMMARY OF STEEL RESULTS

- At 30°C on stainless steel all strains declined by approximately 3 log orders over 49 days, with the veterinary and factory strain surviving better than others
- From day 7 to the end of the experiment at day 35 all of the isolates settled around a log 5 surviving number, representing a 2.5 – 3 log reduction over 35 days at 25° C. The notable exception was the clinical isolate of S. Typhimurium which reached undetectable at day 14.
- At 37°C, the decimal reduction values were very short, with D being 360 minutes for most strains. At day 3, all strains declined to become undetectable.
- Preliminary findings on carbon steel show that all of the isolates settled around a log 4 surviving number, representing a 3 log reduction over 35 days at 25° C. However the factory and veterinary strain of S.Montevideo and the veterinary strain of S.Anatum were undetectable at 14 days.

FRESH CONCRETE

The survival of *Salmonella* on concrete surfaces was investigated, however the initial method of surface inoculating the concrete blocks and recovering them via the swabbing technique and vortexing in saline was not effective, This indicated that either the concrete blocks exhibit antimicrobial properties and *Salmonella* are not able to survive or the method was ineffective in recovering bacteria. The concrete sampling by embedding in agar method, showed clear zones of precipitation around the concrete block from this, the concrete block was converted in to powder and a disc diffusion type experiment was conducted and showed clear zones of growth inhibition.

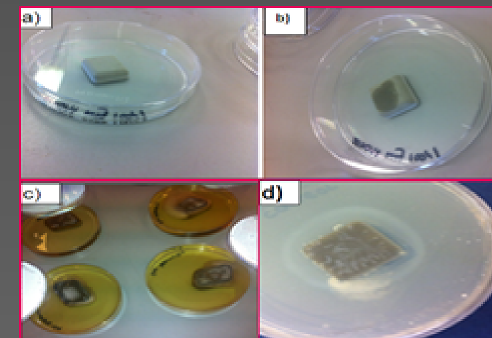

Figure 3: Initial method used; concrete sampling by embedding in agar

a) A concrete block in a sterile petri dish b) concrete block inoculated with *Salmonella* c) Nutrient agar poured around concrete blocks d) clear zones around the concrete block

CONCLUSION

The persistence of *Salmonella* on different surfaces is of great concern to the food industry, results from this study indicate that *Salmonella* survival on stainless steel is affected by conditions in food processing environments, with higher survival rates at lower temperatures. Also several resident factories strains are better survivors on stainless steel compared to clinical and veterinary isolates of the same serotype. Results on other surfaces tested are provisional but there are strong indications that concrete limits bacterial growth. Therefore modelling the factory environment and identifying areas where *Salmonella* can harbour is key in eliminating the organism from food processing environment.