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OÉ Gaillimh
NUI Galway

Salmonella enterica - biofilm formation and survival of disinfection treatment on food contact surfaces.

A Thesis Presented to the National University of Ireland, Galway

for the Degree of Doctor of Philosophy

By

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Ph.D. Thesis - Volume 1

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List of Abbreviations

AFM	Atomic Force Microscopy
ASTM	American Society for Testing Materials
ATCC	American Type Culture Collection
ATP	Adenosine-5'-Triphosphate
°C	Degrees Celsius
CBR	CDC Biofilm Reactor
CDC	Centers for Disease Control and Prevention
CEN	Européen de Normalisation
CFU	Colony Forming Units
CHX	Chlorohexidine
CIDR	Computerized Infectious Disease Reporting
CLSM	Confocal Laser Scanning Microscopy
CSG	Curli Subunit Genes
DAFF	Department of Agriculture, Fisheries and Food
DT	Definitive Phage Type
EFSA	European Food Safety Authority
EPS	Extracellular Polymeric Substances
g	grams
HMDX	Hexamethyldisilizane
HPA	Health Protection Agency
HPCS	Health Protection Surveillance Centre
K	1000
kHz	KiloHetz
kV	KiloVolts
LB	Luria Bertani
Log_{10}	Logarithmic value to the power 10
M	Moles
MBEC	Minimum Biofilm Eradication Concentration
mg/L	milligrams per litre
MIC	Minimum Inhibitory Concentration

MLST	Multi Locus Sequence Type
NARMS	National Antimicrobial Resistance Monitoring
N* or *n	Number
NSSLRL	National *Salmonella*, *Shigella* and *Listeria* Reference Laboratory
O-Ag	O-Antigen capsule
OD	Optical Density
OD_{590}	Optical density read at 590nm wavelengths
P* or *p	*P* values
PBS	Phosphate Buffered Saline
pdar	Pink Dry and Rough morphology
PFGE	Pulse Field Gel Electrophoresis
ppm	Parts Per Million
PT	Phage Type
PVC	Polyvinylchloride plastic
Ra	Mean Surface Roughness
rdar	Red Dry and Rough Morphology
rpm	Rates Per Minute
saw	Smooth and White morphology
sbam	Smooth Brown and Mucoid morphology
SD	Standard Deviation of the Mean
SE	Standard Error of the Mean
SEI	Secondary Electron Imaging
SEM	Scanning Electron Microscopy
Spp.	Species
Subsp.	Subspecies
TAFI	Thin Aggregative Fibres
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UK	United Kingdom
US	United States
%v/v	Percentage Volume
XLD	Xylose Lysine Deoxychocolate

Summary of Content

A recent report indicated that *Salmonella* was the most frequently identified agent in food-borne outbreaks in Europe. *Salmonella* can survive on surfaces and act as a source of cross-contamination. Biofilm formation may allow *Salmonella enterica* survival on surfaces and persistence in food processing environments for extensive periods of time.

In 2008 there was a large food-borne outbreak of *Salmonella* Agona linked to a food processing facility. Prior to this outbreak, *S*. Agona was less frequently implicated in food-borne infections. The *S*. Agona strain SAGOXB.0066 and a variant strain remained in the environment for an extensive period of time. This lead to the hypothesis that the *S*. Agona strain may have a high propensity to form a biofilm and withstand disinfection treatment.

The objectives of this research were to investigate if *S*. Agona SAGOXB.0066 may form a more dense biofilm than other *S. enterica* strains. Secondly, to investigate if *S. enterica* biofilm density increased over an extended period of time. Thirdly, to examine if *S. enterica* biofilm results in enhanced bacterial survival post disinfection treatment.

The results indicate there was no evidence to suggest that *S*. Agona SAGOXB.0066 or the variant strain formed a more dense biofilm than other *S*. Agona strains after 48-hours. However there is some limited evidence to suggest that the serovar *S.* Agona may from more dense biofilm than other serovars. The results also indicate that *S. enterica* strains were able to withstand disinfectant treatment after biofilm formation.

A number of *S. enterica* biofilm studies have tended to examine a limited number of strains or a single biofilm substratum. This research enhances the understanding of the *S. enterica* biofilm formation through the use of multiple strains. Moreover, the results illustrate the extent to which variation in repeated measurements on the same strain or the impact of different biofilm development models may contribute to apparent difference between strains examined.

Declaration

This work is submitted to fulfil the requirements of the degree of Doctor of Philosophy at the National University of Ireland, Galway.

No part of this thesis has been previously submitted at this or any other university.

Apart from due acknowledgements, it is entirely my own work.

Signed: ______________________

Date: ______________________

"To show your true ability is always, in a sense, to surpass the limits of your ability, to go a little beyond them: to dare, to seek, to invent, it is at such a moment that new talents are revealed, discovered, and realised"

Simone de Beauvoir

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Chapter 1

An introduction to *Salmonella* and biofilm formation

1.1. *Salmonella*

Salmonella is a gram negative bacillus, motile through the presence of surface structures called flagella. The complex structure of the *Salmonella* bacterium and its characteristics provide the basis for the accepted naming scheme. This is achieved through serotyping based on the antigenic structure of the *Salmonella* cell surface. The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. According to the current White-Kauffmann-Le Minor Scheme *Salmonella enterica* is further divided into six subspecies (*S. enterica* subspecies *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI) [1]. Fermentation of substances allows the differentiation into the particular subspecies. There are currently over 2,600 *Salmonella* serotypes [2], however 99% of these serovars are in *S. enterica* and almost 60% belong to *S. enterica* subsp. *enterica* (I)[2]. The main serovars discussed in this thesis are *Salmonella enterica* subspecies *enterica* serovar Agona (*S.* Agona), *S.* Typhimurium and *S.* Enteritidis.

Table 1.1: Organisation of *Salmonella* species and subspecies

	Species	**Subspecies**	**Number in each serovar**
Salmonella	*enterica*	subspecies *enterica*	1547
Salmonella	*enterica*	subspecies *salamae*	513
Salmonella	*enterica*	subspecies *arizonae*	100
Salmonella	*enterica*	subspecies *diarizonae*	341
Salmonella	*enterica*	subspecies *houtenae*	73
Salmonella	*enterica*	subspecies *indica*	13
Salmonella	*bongori*		23

Table 1.1 describes the organisation and the number of serovars in each *Salmonella* subspecies as described by Guibdenche *et al.* 2010 [2].

1.2. History of typhoidal *Salmonella*

Salmonellosis has been a human health problem for centuries. In 1880, Carl Eberth first discovered the “typhoid bacilli” through pathological examination of a contaminated spleen. This was followed by the successful isolation and cultivation the “Bacillus typhosus”, now referred to as *Salmonella enterica* serovar Typhi, by Georg Gaffky in 1884. The decline in cases of typhoid fever in the developed world over the last century correlates with the increase in treatment of drinking water, pasteurization of dairy products and the introduction of measures to avoid contact with faecal waste in the food chain [3]. Typhoid fever is no longer endemic in developed countries, however infection with other serovars of *Salmonella enterica* often referred to collectively as non-typhoid *Salmonella* (NTS) remains a major public health challenge.

1.3. Non typhoidal *Salmonella*

There are over 2,600 serovars of non typhoidal *Salmonella*. It is estimated that 90% of the *Salmonella* cases reported are accounted for by 30 serovars. In 2010, *S*. Typhimurium and *S.* Enteritidis accounted for 45% and 22% of the salmonellosis cases reported in Ireland [4]. It is estimated that between 90-95% of non typhoid salmonellosis arises from consumption of contaminated food-stuffs including poultry and other meat products [5]. Therefore vaccination of live animal carriers may result in a reduction of the pathogen in the meat post slaughter. The introduction of a vaccination programme for animals against commonly associated serovars such as *S*. Enteritidis in poultry has proven successful in the past [6].

However, there are drawbacks to the use of a vaccination programme. Firstly some animals may be healthy carriers or asymptomatic therefore vaccination needs to encompass all animals in a herd to be successful. This

may impose enormous costs on farmers due to the large-scale or intensive farming practices frequently used. Additionally, vaccinated animals may also shed the pathogen for weeks after vaccination, therefore there is still a possible risk of entry into the food chain through cross contamination. Moreover, *Salmonella* vaccine development is expensive due to the serovar specific nature of vaccine design and as a result development is restricted to a small number of serovars. Consequently, it may be possible that other serovars may replace the serovars vaccinated against [6]. The displacement of a predominant serovar post vaccination and increased bio-security measures has previously been reported with *S*. Enteritidis replacing *S*. Gallinarum and *S*. Pullorum dominance in poultry [7].

1.4. Clinical features of salmonellosis

The infectious dose of *Salmonella* necessary to induce infection is dependent on multiple factors including virulence of the serovar and host-specific factors such as age, health and host response to the bacterial infection [8]. Following a review of clinical studies performed with participants administered doses of live *Salmonella,* Blaser *et al.* reported that the infectious dose ranged from just 25 cells of *S.* Sofia and *S.* Bovismorbificans, to $2x10^9$ of *S*. Typhimurium to induce salmonellosis and a dose of $1x10^{10}$cells of *S*. Pullorum were necessary to induce salmonellosis in human hosts [9].

The clinical features of a *Salmonella* infection can include self limiting gastroenteritis and dehydration. The symptoms of non typhoid salmonellosis can include diarrhoea, stomach cramps, headaches and nausea. Severe infections including infections in immunocompromised patients can lead to bacteraemia, meningitis, and possible death.

1.5. Treatment of *Salmonella* infection

The most fundamental point about treatment of NTS is that most cases need to maintain hydration and do not need or benefit from antimicrobial treatment. Antimicrobial agents are required for those with invasive disease and for other specific categories [10]. Antibiotics are commonly prescribed for early treatment of *Salmonella* infections. However increasing antimicrobial resistance associated with *Salmonella* strains has hindered effective treatment in the past as reviewed by Sirinavin *et al.* [10].

1.6. Sources of *Salmonella* contamination

Salmonella is a bacterial zoonotic pathogen that is commonly found in the gut and intestinal tract of animals including farmed, domestic and wild animals. Therefore there are numerous routes that allow its entry into the food chain. During the processing of raw meat, contaminated digestive tract contents can colonize surfaces or other meats leading to cross contamination of entire surfaces or products. *Salmonella* can also contaminate fresh fruit and vegetables through contaminated faecal content polluting freshwater and soil. This may arise through fertilization with contaminated animal manure or poor sanitary conditions and waste water treatment. Herman and colleagues reported that there was a 30% increase in the number of *Salmonella* outbreaks associated with contamination of leafy greens despite a decrease in consumption over a ten year period [11].

Live poultry at all stages of the farming process can be carriers of *Salmonella*. *Salmonella* can also colonize the ovaries of infected poultry leading to colonized eggs produced via transovarial transmission [12]. *Salmonella* can also contaminate egg contents when contaminants remain on the outside of soiled eggshells or seep through the surface through

cracks or penetration of weak eggshell membranes [13]. Dairy products can become contaminated with *Salmonella* through poor handling practices at the farming level (such as milking of animals). Bacterial contamination in dairy products can be eliminated by pasteurization.

1.7. Predominant serovars implicated in food-borne outbreaks

A recent report published by the European Food Safety Authority indicated that that *Salmonella* was the most frequent recognised agent involved in food-borne outbreaks [14]. Certain serovars are predominant in particular foods and animal sources, therefore identifying the serovar which causes contamination is valuable in identifying the source outbreaks of food-borne origin. Moreover, particular serovars are host specific for example *S*. Gallinarum in poultry [6], *S*. Abortusovis in sheep [15], *S*. Abortusequi in horses [16].

Over the years *S*. Typhimurium was the predominant cause of human food-borne salmonellosis in Europe and in the United States [7]. However since the emergence of *S*. Enteritidis into the food chain in the 1970-1980's, there has been significant displacement of dominant serovars and *S*. Enteritidis is reported more frequently than *S*. Typhimurium in many European countries [5].

1.8. Serotyping of *Salmonella*

Salmonella serotyping is based on the immunoreactivity of the two surface antigen groups, the O antigens (somatic antigens) and the H antigens (flagellar antigens) and to a lesser extent the Vi antigens (capsular antigens) which are present in very few serotypes (*S*. Typhi, S. Paratyphi C and *S*. Dublin). The O antigens are the carbohydrate (O subunits or

polysaccharides) component attached to the core oligosaccharide of the lipopolysaccharides molecule. Variation between O antigens can occur as a result of differences in the sugar component of the O-subunit, from the bonds between the sugar components that form a subunit or the linkage of the subunits [17]. The O antigens can be divided into two groups the O-group antigens (core antigens) and the ancillary antigens (additional polysaccharide subunits that are added to the core antigen structure). Each O antigen has been designated a number for identification. Strains that do not express O antigens are referred to as rough in the antigenic structure details [17]. Historically some were assigned alphabetically and the terms are still in use [17]. All O antigens detected on the surface are listed sequentially following the White-Kauffmann-Le Minor scheme [1].

The H antigen is the filamentous portion of the flagellar component of the bacterium. The antigenic differentiation is related to diversity in the middle portion of the flagellin protein. Most *Salmonella* cells can express two different H antigens (diphasic). The phase 1 antigen is encoded by the *fliC* gene and the phase 2 antigen is encoded by the *fliB* gene [17]. Most cells only express 1 antigen at a single time. Cells that can only express 1 antigen are referred to as monophasic, which can occur naturally in some serovars or through loss of either the *fliC* gene or *fliB* genes in serovars that are usually diphasic such as *S*. Typhimurium [17]. The lack of a phase 1 or phase 2 antigen is denoted by a "-" in the antigenic structure as described in *S*. Typhimurium in section 1.9.2. Non motile strains do not express phase 1 or phase 2 antigens and are denoted with two "-" in the antigenic formula.

The O antigen is detected in a bacterial suspension taken from an agar plate. Detection of H antigens is performed on a bacterial suspension from broth (to ensure motility of the bacterium). The presence of antigens is

detected by agglutination tests on glass slides with the corresponding commercial anti-sera.

1.9. Methods used for epidemiological surveillance of *Salmonella*

Bacteriophages (phages) are viruses that can only grow or replicate within a bacterial cell. Phage typing can differentiate between strains of the same serotype based on the principle that certain phages will only lyse particular strains of a specific serotype. The lysis pattern can be compared to a standard scheme for each serovar to determine the phage type of the strain [18]. The typing schemes were devised around particular serovars of *Salmonella* have proven very useful epidemiological tools for investigating outbreaks of *S*. Typhimurium [19], *S*. Enteritidis [20] and *S*. Agona [21]. When used in conjunction with antimicrobial susceptibility testing, phage typing has led to identification of a number of large international outbreaks including multi-state outbreaks of *S*. Typhimurium DT104 [22].

Pulsed Field Gel Electrophoresis (PFGE) was adapted for *Salmonella* in the 1990's and is still considered as the "Gold Standard" for supporting the identification of epidemiological links between isolates [23]. Although multilocus sequence typing (MLST) and whole genome sequencing (WGS) are quickly becoming the more favoured options [24]. The procedure is based on cutting the intact bacterial chromosome with site-specific proteins (restriction endonucleases). The DNA fragments are then separated using pulsed currents (electrophoresis separation) to generate a banding pattern that forms the basis for assessing similarity between isolates. However a major disadvantage with using PFGE to distinguish between *Salmonella* strains is it has low discriminatory power for certain serovars [25]. Other disadvantages include inter-laboratory variation and reliance on reader interpretation of banding pattern.

Multi Locus Sequence Typing schemes (MLST) are used to identify unique DNA sequences or alleles from a portion of housekeeping genes of the organism under examination. The unique DNA sequences are assigned a unique sequence type number which can be compared to a database of assigned sequence types of the organism under examination. This method has been used to identify alteration in housekeeping genes and to determine the order and sequence of evolution within a species over time [26].

Typing methods have recently progressed to the level where multiple strains can be analysed to identify a large number of single nucleotide polymorphism (SNP) through whole genome sequence analysis. This method can differentiate between strains within the same serotype, with a similar pulse field profile and phage type based on the sequence type of the strain [27].

1.10. Zoonotic Infection - transmission from farm to fork

Salmonella adherence to epithelial cells and the formation of intricate biofilm communities may play an important role in the carriage of the organism in animals [28]. Animals can be asymptomatic carriers of *Salmonella* in their intestinal tract therefore hygiene precautions are always required when handling carcasses and processing raw meat. Contamination of food such as meat can stem from poor handling of raw meat or cross contamination originating from the intestinal content of livestock.

Once surfaces have become colonized with *Salmonella*, the pathogen can continue to contaminate food and food processing surfaces further down the processing line. Barker and colleagues demonstrated that during the processing and handling of contaminated raw meat, work surfaces can

become colonized with >1000 CFU/ 5cm^2 of *Salmonella* [29]. Moore et al. also demonstrated that once a surface is colonized, *Salmonella* can readily transfer onto food products [30]. Subsequently contamination can result in food-borne infections if food is undercooked or incorrectly preserved (smoked, salted). Previous research has also shown that during handling and preparation of raw chicken (contaminated with *Salmonella*) that domestic kitchen surfaces, including chopping boards, utensils, taps and sink rims, fridge and oven doors can become colonised with the bacterium, which can still be present after cleaning with disinfectants [31]. It is estimated that cross contamination (from carcasses to surfaces and vica versa) in the slaughter process can account for 29% of the total of *Salmonella* contaminated carcasses [32].

The presence of bacterial cells persisting after cleaning may also lead to the development of a biofilm on the implicated surfaces and result in potential for recurring contamination events. Numerous food-borne outbreaks of *Salmonella* have been linked to contaminated processing areas including a processing line [33-34], baking utensils [35] and in food storage containers [36].

1.11. Salmonellosis in Ireland

The Computerized Infectious Disease Reporting system (CIDR) was established in 2004 to facilitate the collection of data relating to infectious diseases in Ireland [37]. The number of cases of *Salmonella* submitted to the National *Salmonella, Shigella* and *Listeria* Reference Laboratory (NSSLRL) for typing have declined by over 10% in the past 6 years. The two predominant serovars associated with human salmonellosis reported in Ireland were *S*. Enteritidis (18-41%) and *S*. Typhimurium (23-31%)[38] . This trend is also similar to other European countries [39]. *S*. Enteritidis was the

most common serovar submitted to the NSSLRL, however there has been a switch in dominance and for the past four years and *S*. Enteritidis has been replaced by *S*. Typhimurium as the most dominant serovar. In most European countries *S*. Enteritidis remains the dominant serovar [39]. There has also been increasing number of the monophasic variant of *S*. Typhimurium which is further discussed in section 1.9.2 of this chapter [38].

Interestingly, while the incidence of predominant serovars such as *S*. Enteritidis and *S*. Typhimurium has began to decline in recent years, there has been a slight increase in the global reports of other serovars including *S*. Agona [40]. Through the use of the World Health Organization Global Foodborne Infections Network, Hendriksen *et al.* found that the number of cases of *S*. Agona were increasing while the predominant serovars *S*. Enteritidis and *S*. Typhimurium were decreasing between 2001 and 2007 [40]. The percentage of *S*. Enteritidis reported fell from 44.2% of reported cases to 41.5% (p<0.01) while the percentage of *S*. Typhimurium fell from 18.9% to 15.0% (p=0.16). Overall, *S*. Agona increased from 0.08% to 1.5% between 2001 and 2007, this trend was seen in both developed and developing regions reporting cases [40].

1.12. *Salmonella* Agona

1.12.1. *Salmonella* Agona – History of food-borne outbreaks

Salmonella enterica subspecies *enterica* serovar Agona has the full antigenic formula 1,4,[5],12 f,g,s [1,2] [z27],[z45] according to the White-Kauffman-Le Minor scheme [1]. The antigens present on *S*. Agona include the somatic O antigens 1,4,[5],12 of which "1" is only present if the bacterial culture is lysogenized by the corresponding phage agent. The factor "[5]" denotes that the factor may be present or absent without phage conversion. *S*. Agona contains seven flagellar or H-antigens, three phase 1 antigens-f, g, s, two phase 2 antigens [1,2] and two additional H

antigens [z27],[z45] [1]. The antigenic formulae of the *S*. Agona associated with this outbreak was 4,12:f,g,s as the two phase 2 antigens were not present.

Salmonella Agona was first isolated in central Ghana [41]. It was rarely identified again until a spate of outbreaks 10 years later in Israel, Netherlands, the United Kingdom (UK) and the United States (US) which were traced back to imported fish meal and animal feed [42]. It is suspected that *S*. Agona made its way into the human food chain due to persistence in animal livestock. Ever since its successful establishment in the food chain it has been responsible for a number of large outbreaks worldwide [43].

According to the figures released by the Irish Department of Agriculture and Fisheries (DAFF) *S*. Agona was the third most frequently isolated serovar from food samples when samples were reported between 2006 and 2011 [4]. However the number of *S*. Agona samples decreased dramatically from 87 in 2009 to 26 samples in 2010 and 4 samples received in 2011. Therefore this ranking may reflect the large outbreak of *S*. Agona recorded in Ireland over this time (2008-2009). However the number of samples received in previous years were also high (93 samples received in 2007). This particular outbreak is discussed in further detail in section 1.9.2 of this chapter. Douris *et al.* also reported that the number of *S*. Agona samples received through the United States National Antimicrobial Resistance Monitoring System (NARMS) also increased and *S*. Agona was in the top 15 serovars reported in animals between 1997-2003 [44]. *S*. Agona was also in the top 20 isolates associated other sources including clinical and non-clinical samples [44].

1.12.2. Previously reported *S.* Agona outbreaks

There was a dramatic increase in the number of cases of *S*. Agona reported in Israel in 1995-1996, with 2200 cases reported in a 5 month period [45]. However an epidemiological investigation failed to identify a link with the food chain until authorities received a call from the health authorities in the UK reporting that there was an outbreak of *S*. Agona which they suspected to have come from ready to eat kosher savoury snacks imported from Israel [45]. The international outbreak, with cases reported in the UK, US and Canada was first noticed as all the reported cases were from children with "Jewish sounding surnames" [46]. The link to the food product had not previously been traced back to the snack in Israel as the snack was consumed by most young children in Israel. The contaminated snacks were manufactured on at least 7 occasions over a 4 month period which suggests a prolonged fault in good manufacturing practices within the food processing plant [46]. During an environmental investigation of the food plant *S*. Agona was not detected [45]. However the contaminated product contained high quantities of bacterial strain responsible for the outbreak (2-45 organisms per 25g packet of produce) [46]. PFGE was used to assess the genetic relatedness of the samples from the UK, US, Canada and France. The results of the investigation indicated that the strains were closely related (indistinguishable PFGE pattern) [47].

In 1996, a smaller outbreak of *S*. Agona PT15 (6 confirmed cases) was reported in the UK. The outbreak was associated with the consumption of undercooked turkey meat. After an investigation, the contamination was suspected to stem from deficiencies in raw meat handling, preparation and appropriate cooking practices [48]. In 1996 there was also an outbreak of *S*. Agona in the state of Texas, US. This outbreak was responsible for eighteen salmonellosis cases involving improperly prepared machacado (air dried raw beef product) in two restaurants [49].

S. Agona has also been implicated in two large consecutive outbreaks associated with contaminated powdered infant milk formula produced in France. The outbreaks which were traced back to a contaminated production line [50]. These outbreaks were responsible for a total of 141 confirmed cases in infants less than 12 months of age. The outbreaks were identified by the public health department due to a sudden increase in reported cases of *S*. Agona from January to February 2005, in comparison to the usual low incidence of *S*. Agona cases reported [50]. In a similar situation in Germany, the authorities noticed an increase in the number of cases of *S*. Agona over a two month period of January to February 2003, the reported cases were almost entirely in infants less than 13 months of age. Through an epidemiological investigation, *S*. Agona was traced back to 12 aniseed containing herbal teas that had been imported from Turkey. It was suspected that the aniseeds may have been contaminated by the use of manure as a fertilization agent [51]. A total of 40 cases of *S*. Agona were linked to the contaminated aniseed (through PFGE profile).

The largest *S*. Agona outbreak reported in the US was due to contaminated "Malt-o-meal" toasted oat cereal, which involved over 200 cases in 11 American states [52]. An epidemiological study traced the contaminated food back to the food processing plant where an extensive environmental study was performed. *S*. Agona was found in low levels throughout the plant including the floor, production equipment and the exhaust system in the plant [34, 53]. In addition to the contaminated surfaces, the health authorities also found that the insanitary condition of equipment, poor staff preparation practices, and an incorrect water supply system were factors which could have lead to the outbreak [54]. Subsequent to the *S*. Agona outbreak in "Malt-o-Meal" cereal in 1998, there was an additional outbreak 10 years later, with an unsweetened puffed rice and wheat cereal. After prompt identification, notification to the authorities and product recall this outbreak was notably smaller than the first, with a total of 28

cases reported in 15 states. Nevertheless, the persistence of a particular strain of *Salmonella*, despite cleaning and interventional work, suggests that a biofilm may be responsible for its survival. Following an outbreak investigation, Russo *et al.* speculated that it is possible that the *S*. Agona strain (PFGE profile JABX01.0001) persisted in wall cavities of the food processing plant for the ten year period and was released back into the food processing environment via dust particles during construction work prior to the second outbreak [55]. The persistence of *Salmonella* Agona in fish meal and processing plants despite intensive cleaning and ongoing monitoring has also been documented elsewhere [56].

1.12.2.1. The *Salmonella* Agona SAGOXB.0066 outbreak

In July of 2008, the National *Salmonella Shigella* and *Listeria* Reference Laboratory Ireland (NSSLRL) noticed an increase in the cases of a *S*. Agona (a cluster of 6 were seen over a fortnight, where 10 had been the average reported annually previously). The NSSLRL reported these findings to the Health Protection Surveillance Centre (HPSC) who alerted the UK authorities after which a review of *S*. Agona reported cases in the UK was performed. The Health Protection Agency (HPA) identified 32 isolates of *S*. Agona with a Phage Type PT39 which occurred in April in England. Scotland also identified 15 *S*. Agona which were indistinguishable to the Irish strains using Pulse Field Gel Electrophoresis. As a result of these findings, an international outbreak was declared on the 18th July 2008 [57].

During an investigation of the NSSLRL database, all cases of *S*. Agona were re-examined, PFGE was performed and the outbreak strain referred to as SAGOXB.0066 was identified. Through the Irish Department of Agriculture, Fisheries and Food (DAFF), the outbreak investigation team were able to trace the outbreak strain to an Irish food production company (referred to in the published report as company A) [58]. The outbreak strain had been

isolated from undercooked bacon produced by the Company (Company A). The undercooked meat had come as a result of a cooker failure on one of their production lines, the bacon was subsequently quarantined, however at this point, the contaminated undercooked products had already entered the food chilling area, where it may have come into contact with surfaces or other meats [58].

The food processing plant (Company A), supplied two chain stores (Chain A and Chain B) implicated through patient interviews. Therefore the hypothesis was that the breakdown of a cooker in the food processing plant may have allowed the pathogen to remain viable in the cooked meat food supplied by the company. After inspecting the food processing plant and the outlets of the two chain stores, multiple samples of beef strips from Company A supplied to Chain A were found positive for the outbreak strain in Northern Ireland, Wales and Ireland. Bacon pieces from chain B which were sourced back to company A were also found to be positive for the outbreak strain. In addition to the microbiological testing of the food, extensive environmental testing was performed within the food plant. The outbreak strain was isolated in the low risk area in the environment (raw food area) during the outbreak which may have allowed its travel into the high risk area (cooked, ready to eat area). All contaminated products (beef and bacon) were withdrawn from the market, in addition nine other products that may have come in contact with the contaminated batches were also recalled. Production and circulation of food products ceased until all health authorities were assured it was safe to restart. A pharmaceutical grade clean (using vaporised hydrogen peroxide) was applied throughout the plant and bio-markers were used to detect any post cleaning contamination. All personnel were also tested for carriage of *Salmonella* but none was detected [58].

Company A was a major exporter of processed foods throughout the EU, which may provide an explanation as to how the outbreak disseminated so

rapidly throughout Europe. In total 163 cases of the outbreak strain of *S*. Agona SAGOXB.0066 were reported involving 10 countries over a 10 month period including eleven cases in Ireland, 143 cases in the UK [England (*n*=96), Wales (*n*=11), Northern Ireland (*n*=2), Scotland (*n*=34)] and nine cases in mainland Europe [France (*n*=3), Luxembourg (*n*=2), Sweden (*n*=2), Finland (*n*=1) and Austria (*n*=1)] . The infection was quite severe as 25 patients were hospitalized and 2 elderly patients died [57-58].

Through an investigation of previous *S*. Agona isolates, it was noted that the outbreak strain SAGOXB.0066 had been circulating in Ireland for the previous 3 years and was associated with one sporadic human salmonellosis case reported in 2005. In 2005, the outbreak strain had previously been found in a poultry processing plant with additional strains found in broiler carcasses and the poultry farm that supplied the poultry processing plant. The outbreak strain was also identified in milk filter residues in cork in 2003 and in goat's milk filters in Monaghan in 2005 [58]. Even though the outbreak strain has been detected in the food chain for 3 years, there were no outbreaks prior to 2008. The outbreak strain was also detected in river water samples during the outbreak [57-58].

1.12.2.2. The variant strain – remnants of SAGOXB.0066

Continuous environmental swabbing performed both inside and outside the plant revealed a variant of the strain was persisting in drains of the plant for an extended period of time despite intensive cleaning and disinfection (personal communication with members of the investigation team [58]). The persistence of *Salmonella* (including *S*. Agona), despite intensive cleaning may be indicative of biofilm formation [56]. The differences in strains between the outbreak strain and the variant are visible through PFGE, see image provided by the NSSLRL (see appendix 3).

1.13. *S*. Typhimurium and "*S*. Typhimurium-like" strains

The *S*. Typhimurium strains studied have an antigenic structure 4,5,12;i:2 and its monophasic variant 4,5,12;i:- [1]. Together these antigenic classifications accounted for 35% of all cases of *Salmonella* submitted to the NSLRL in 2009 for typing [59]. In recent years there has been a dramatic increase in the reported cases of the monophasic serovar 4,5,12:i:-, this phenomenon has also been seen in many other member states in Europe [60]. Two strains of *S*. Typhimurium studied in this research have a PFGE profile STYMXB.0131, this PFGE profile has been implicated in numerous outbreaks throughout Europe with the number of reported cases increasing by 321% in the UK and 414% in France over a 4-5 year period [60]. In a collaborative report of EU states, Hopkins *et al.* identified the PFGE profile STYMXB.0131 in 28% of monophasic Typhimurium strains assessed (*n*=114). This PFGE profile was previously associated with *S*. Typhimurium with the definitive phage type DT193 and phage type PT507 in Finland, the Netherlands and the UK [60]. The monophasic *S*. Typhimurium was initially traced to pork products, however it has also been found in poultry, cattle, non-domestic birds, broilers and humans [61]. Due to the rapid dissemination of monophasic *S*. Typhimurium the research described in this thesis also investigated if this variant had distinctive properties that enhanced biofilm formation on contact surfaces, although it is deemed as typically very similar to *S*. Typhimurium at a molecular level [61]. The PFGE profile for the two STYMXB.0131 strains is also provided courtesy of the NSSLRL (see Appendix 3).

1.14. *Salmonella enterica* serovar Enteritidis

Salmonella Enteritidis has the antigenic structure of 1,9,12 g, m –; [1]. Although S. Enteritidis has been reported in poultry as far back as 1930’s, up until the 1970s, there were relatively few reports in poultry, eggs or in human cases [6]. As previously discussed, it is suspected that vaccination

and culling of seropositive hens in order to reduce *S*. Pullorum and *S*. Gallinarum may be responsible for creating a niche to allow *S*. Enteritidis to become established in poultry [7]. It is also hypothesised that the ability of *S*. Enteritidis to infect poultry without causing disease may have aided its successful dissemination and development as a predominant strain in poultry . However since its introduction into the food chain in the 1970's *S*. Enteritidis has also been linked to food borne outbreaks in beef [62], pine nuts [63], almonds [64] and sprouts [65]. *Salmonella* Enteritidis is now the most frequently reported serovar associated with in human salmonellosis cases in Europe [14]. In recent years there has been an increase in the number of cases of *S*. Enteritidis classified with the phage type 14b. This strain variant (14b) had also been implicated in over 443 cases including 14 outbreaks in the UK since August 2009 [66]. *S*. Enteritidis outbreak strain with the phage type 14b was associated with consumption of eggs. Previous research has suggested that *S*. Enteritidis biofilm formation on conveyor belts may be responsible for cross contamination in an egg processing plants [67].

1.15. Biofilm

A biofilm can be described as a community of cells, mixed species or mono-species which is attached to a surface and/or to each other. Biofilm cells produce proteinaceous substances that allows synergic growth and protection from possible harsh environments it may encounter [68]. In the seventeenth century, a Dutch scientist Van Leeuwenhoek was the first person to discover biofilm cells which he described as "animacules" on his dental plaque. This discovery was further investigated and characterized in 1978 when it was found that the bacteria within a biofilm grow within a matrix that allows them to attach tightly to a surface and behave differently to their planktonic variants [69]. When investigating the aquatic

ecosystem with the use of microscopy, it was deduced that 99.9% of the bacteria assessed grew in a biofilm on a range of surfaces [69].

The formation and structure of a biofilm is influenced by numerous variables including species [70], surface [71], nutrients available [32, 72] and other environmental conditions [73]. The biofilm development process is initiated with single cells attaching to a surface or to each other, this is then followed by the formation of clustered cells or microcolonies [74]. Over time, the microcolonies are surrounded by a protective layer of protein-rich substances referred to as extracellular polymeric substances (EPS). As the biofilm grows the biofilm matrix develops water channels that act as a support system to provide nutrients to the less exposed areas of the biofilm and act as a route for waste disposal [74].

Previous research has suggested that almost all bacteria can form a biofilm and that once the transition from planktonic cells to their biofilm state is initiated, this becomes the optimum form for the bacterial cells existence [75]. It is also reported that single-species bacteria can be heterogeneous in their nature, with each group of cells undertaking a different role in the biofilm development depending on environmental cues such as availability of oxygen. The cells of a single species within a bacterial culture may display differences in metabolism, gene expression, morphology and physiology allowing it to work well within its community, depending on it spatial relationship in the biofilm matrix [76]. For example, a bacterial cell embedded in the biofilm matrix may survive on less nutrients and lower oxygen gradients by going into a dormant state [75].

1.15.1. Structural components and genetic cues to biofilm development

Microarray studies have identified genes responsible for changes between planktonic cells and their biofilm counterparts, although when assessing the extent of the changes particular consideration has to be given to the conditions under which the experiments are performed [75]. The development and genetic signalling pathways involved in a *Salmonella* biofilm formation are complex. There are four major components to the structure of the *Salmonella* biofilm: curli, cellulose, capsular polysaccharides and lipopolysaccharides.

Curli fibers, previously referred to as thin highly aggregative fibers (Tafi) are one of the main components of the extracellular polysaccharide (EPS) matrix [77]. Curli have been associated with numerous processes in biofilm development including host invasion, colonization, cell-to-cell contact and increased motility [28]. The proteinaceous aggregative fimbriae referred to as curli are formed as a result of the transcriptional regulator CsgD (curli subunit gene), which was previously referred to as *agf*D in *S*. Typhimurium. CsgD allows the activation of the *csgBAC* and *csgDEFG* operons [78]. The expression of this subset of genes can be identified in multiple species within the *Enterobacteriaceae* family, however the role and expression can vary depending on the strain [78]. The expression of curli is hypothesised to respond to environmental cues such as changes of temperature (optimally expressed <30°C, oxygen tension (microaerophilic), presence of ethanol stress, alteration of osmolarity (increased salt) or pH [79]. The curli subunit genes are optimally expressed during the stationary phase of bacterial growth, with the stationary-phase protein sigma factor RpoS (σ^S) enabling the process [80]. Previously published research has demonstrated the pivotal role of RpoS in the stress response of *S. enterica* [81].

Cellulose is a glucose polymer of the extracellular matrix which acts as a "sticky component" which facilitates the bacterial attachment to surfaces

[82]. Previous research has suggested that cellulose plays a crucial role in biofilm formation including in batch biofilm reactor systems [82], epithelial cell surfaces [83], glass cover slips [84] and alfalfa sprouts [85]. However others have suggested that cellulose is not crucial for *Salmonella* biofilm formation on gallstones [84] and polystyrene [86]. The activation of cellulose production comes as a result of the regulator AdrA. AdrA stimulates cellulose formation via the bacterial cellulose synthesis operons *bcsABXC-bcsEFG* [82]. Römling hypothesised that the production of cellulose is subject to selective pressure and under certain conditions it is not produced in host-invasive diseases (in less common serovars). Römling *et al.* suggested that as cellulose is not always found in strains isolated from invasive diseases, this may indicate that selective pressure may prevent the production of cellulose under certain invasive conditions. Römling *et al.* also suggests that the production of cellulose by strains such as *S*. Enteritidis and *S*. Typhimurium outside of an animal host may allow the bacteria to survive in other environments [87]. The hypothesis that cellulose is not necessary for virulence has been supported through *in vivo* studies elsewhere [82].

Curli and cellulose combined form the extracellular polymeric substances (EPS) or matrix of a developed biofilm [78]. It is suggested that curli provide the thin rigid link between cells whereas cellulose provides more stable attachment through elastic polysaccharide bonds [87]. Gualdi *et al.* suggested that *csgD* over expression results in limited production of curli, while deletion of the bcsA, gene responsible for cellulose production resulted in increase of curli-dependent bacterial attachment [88]. Gualdi *et al.* also suggested that both expression of curli and cellulose may be interchanged depending on the requirements of the cell and the host environment [88].

There are three categories of polysaccharides that protect a bacterial cell from harsh surroundings. Namely extracellular polysaccharides (loosely attached slime layer); capsular polysaccharides (tightly attached to the bacterial cell) and the lipopolysaccharides that form the outer structure of the bacterial cell wall. Capsular polysaccharides and lipopolysaccharides can be differentiated through immunoreactivity, charge of the surface and the formation of the capsule structure [89-91]. The O Antigen capsule (O-Ag) is co-regulated with the curli and cellulose associated proteins. The capsule is highly hydrated, similar to other commonly reported capsules in gram negative bacteria and is composed of over 2000 polysaccharide units [91]. The *yihU-yihA* and *yihVW* operons have been recognized as having important roles capsules assembly. The capsular polysaccharide is reported to support bacterial persistence in harsh environments such as desiccation [90]. Unlike curli and cellulose, the O-Ag capsule has not been shown to provide support for structures [90]. While it has been suggested that the O-Ag capsule may have an important role in biofilm formation with *S.* Typhimurium, the capsule was also not necessary for attachment of *S. enterica* cells to glass or polystyrene [28, 92].

In addition to playing a key role in the structure of all Gram negative bacteria in the planktonic state, the lipopolysaccharides (LPS) also play a role in the structure of a biofilm. Prouty and Gunn demonstrated that alteration of *galE* and *rfaD* (genes associated with the LPS and biofilm development) resulted in reduced biofilm formation on glass [84]. The *galE* gene is involved in sugar metabolism (encoding uridine diphospho-galactose-4-eprimase, which is involved in the structural development of the biofilm cells. This research also found that mutants lacking the *rfaD* gene were unable to produce a biofilm on glass due to an impaired ability to add the monosaccharide repeating units to the core oligosaccharides of the LPS. However both mutants were able to form a biofilm on gallstones [84]. Kim and Wei also found through mutational studies, that alterations

to genes involved in flagellar and LPS production (*flg*K, *rfbA, nusA)* resulted in significantly lower biofilm formation by *S*. Typhimurium attachment to stainless steel and glass in meat and poultry broths [93].

1.15.2. Identification through colony morphology

The formation of the extracellular polymeric matrix of a *Salmonella* biofilm varies depending on the method of growth [28]. As discussed some of the more consistently confirmed elements have included components of the thin aggregative fibers referred to as curli, EPS and cellulose. Numerous authors have identified components involved in biofilm formation through assessing the colony morphotype on congo red (CR) agar [77, 86, 94-95]. This method involves growing the test organisms on Luria-Bertani Agar plates with the addition of indicator dyes (congo red, coomassie brilliant blue and calcofluor). The indicator dyes facilitates detection of cellulose and curli fimbriae which play key roles in *Salmonella* biofilm production [77].

The curli and cellulose positive phenotype can be identified as red, dry and rough (rdar) on CR agar [82]. The curli negative phenotype is associated with pink dry and rough (pdar) colonies. The brown dry and rough colony morphology is associated with cells lacking synthesis of cellulose. While in the absence of both curli and cellulose smooth and white (saw) colonies are detected on CR agar [82]. The smooth brown and mucoid (sbam) morphotype on CR agar is indicative of a lack of cellulose and over production of capsular polysaccharides [86]. Efforts have been made to link morphotype of CR agar with capacity to form biofilm. Studies have indicated that the *Salmonella* rdar morphotype accounts for up to 99% of *S. enterica* subgroup I [96]. However, others have questioned its reliability as an indicator of the level of biofilm formation on food contact surfaces [67].

1.15.3. *Salmonella* grown as a biofilm on food contact surfaces

Biofilm on food contact surfaces is a major concern for the food processing industry and has been the subject of significant studies in recent years. Biofilm has been documented on a wide variety of surfaces and the material and microtopography of surfaces may influence biofilm phenotype, structure and density [84, 97-98]. Surface properties may play a key role in biofilm attachment, where properties such as hydrophobicity (and its ability to retain moisture) surface roughness, crevices and cleanability may support biofilm formation [99]. Chia and colleagues found correlation between the surface charge of material and the attachment of *Salmonella* (*S*. Sofia, *S*. Typhimurium, *S*. Infantis and *S*. Virchow) to teflon, stainless steel, glass, rubber and polyurethane using epifluorescence microscopy [100]. Ortega *et al*. also indicated that the surface roughness may contribute to bacterial attachment as the deep crevices (possibly after corrosion using abrasive chemicals) allow bacterial entrapment and provides a niche which allows for protection against shear force and stress conditions [101]. Korbrer *et al.* also found the presence of crevices in contact surfaces allowed protection from disinfectants [102]. The extent of bacterial attachment on surfaces may result in biofilm formation on a surface over time [103].

Salmonella can form a biofilm on numerous surfaces found in food processing environments including glass [104-106], concrete [71], stainless steel [71, 107], silestone [108], granite [107], teflon, silicon and a variety of synthetic plastics [71, 104-105]. Extensive research in Norwegian feed and fish meal factories suggested that the ability of *S.* Agona, *S*. Montevideo, and *S*. Typhimurium to form a biofilm on surfaces increased its persistence in processing environments [56]. *Salmonella* was found to persist in the environment despite intensive cleaning. Positive samples were collected from filters, drains, contact surfaces and from food products [56]. This

observation has also been reported previously. Evans *et al.*, detailed how consecutive outbreaks of *S. Enteritidis* PT4 were traced back to baking equipment [109]. Stocki *et al.* hypothesised that *Salmonella* colonization and persistence on egg conveyor belts can be attributed to the surface type (plastic, vinyl, nylon, hemp-plastic) more than the presence of the rdar morphotype (physiological adaptation commonly associated with *Salmonella* biofilm formation) [67].

1.15.4. Methods for Biofilm analysis

Numerous research projects have focussed on the use of the microtitre plate system for the assessment of biofilm formation due to its high throughput nature and ease of use [56, 86, 96, 110-113]. In brief, the test organisms are grown in a microtitre plate at optimal temperature in the presence of a nutrition source (usually Tryptic Soy Broth or Luria-Bertani Broth) for 2-28 days (depending on experimental design). After the biofilm has formed on the surface the density of cellular attachment is quantified by reading optical density after indicator dye has been added (such as crystal violet). This biofilm method is static (no consistent flow of nutrients) therefore it may not reflect real-life conditions of biofilm in industry where a biofilm may generally be in contact with flow of liquids and nutrients. Also, the surface properties of the polystyrene microtitre plate systems may not reflect the stainless steel, glass and concrete surfaces usually found in many industrial food processing areas [107].

The Minimum Biofilm Eradication Concentration (MBEC) previously referred to as the Calgary device [114] was designed as a suitable alternative to the microtitre plate method. The lid of the plate is set with 96 individual plugs that can fit into the each well of a 96 well microtitre plate. The plugs can be coated with a particular finish of material (i.e.

concrete) and aseptically removed from the lid of the microtitre plate after biofilm formation. The plugs are then used for enumeration via plate counts or for evaluation by direct microscopy. Another high throughput assay for assessing biofilm formation has been devised using a BioFilm Ring Test ®. The biofilm ring test is based on placing magnetic beads (toner) in a high concentration of a test organism, which is then allowed to develop into a biofilm by incubating the organism in a microtitre plate. After the incubation period, the microtitre plate is placed on a magnetic support block [115]. The hypothesis is that if a biofilm is formed it will not allow the magnetic beads to agglutinate at the centre of the microtitre plate [115].

An additional alternative to the microtitre plate system is the insertion of coupons made of the relevant materials into 6/24 well microtitre plates [71, 98]. This method allows biofilm growth and direct visualisation of the process through microscopy. Similarly nutrient media is spiked with a known concentration of bacterial culture and allowed to grow at optimal temperatures. After the biofilm has grown, the coupons can be assessed with microscopy or the viable colony counting though plate count methodology. However this procedure still does not account for sheer stress which can result in greater attachment, increased biomass and higher physiological activity than a biofilm grown in laminar flow or batch-phase models [116-117].

In order to overcome this issue, some researchers have chosen to examine biofilm formation in a modified Robbins device [118], which allows nutrients to flow through the device and come into contact with the surfaces that are inserted into the ports. The device is connected to fresh media via tubing which allows continuous flow of nutrients and waste disposal. The biofilm forms on the surfaces which can then be removed for further analysis (microscopy or plate counts). Similar Plug Flow devices

[119] and flow cell reactors have been adapted and fitted with a glass chambers into the devices to facilitate real-time imaging [102]. This method involves staining the bacterial cells with a fluorescent dye before inoculating the reactor and assessing the accumulation of cells over time using microscopy.

The Center for Disease Control and Prevention Biofilm Reactor (CBR) has been extensively used in recent studies [117, 120-124]. This continuous flow method was chosen for this research as it allows biofilm formation on multiple surfaces simultaneously. The CBR also has the benefits of incorporating sheer stress and examination of spatial growth (through microscopy) and evaluation of viable cells (though plate counts). The CDC reactor was the first model approved by the American Society for Testing Materials (ASTM) for the development of biofilm on surfaces [117]. As of 2012, both the CDC biofilm reactor (ASTM standard number E2871-12)[125] and the MBEC Assay (ASTM standard number E2799-12)[126] have been approved by ASTM. The CBR is discussed in greater detail in chapter two.

1.16. Biocides and Disinfectants

The term biocide is inclusive of a broad range of chemical agents such as disinfectants, antiseptics, herbicides, preservatives, insecticides, fungicides, enzymes and probiotic substances such as *Lactobacillus* species [127-128]. The term biocide does not generally include antibiotics [128-129] which usually target a specific organism by use of a compound at a low concentrations while biocides generally target all organisms through the use of high concentration of the compound. A disinfectant can be classified as a product that reduces the number of viable organisms on a surface [129]. Because of extensive variability in tests it is difficult to develop a

standardised approach to disinfectant and biocide testing. The European Committee for Standardisation have developed protocols for documenting efficacy of products sold within the European Union [130]. According to the European suspension test (EN1040) a 5 $\log_{10}$ reduction in the number of viable cells (99.999%) is necessary to prove the efficacy of a disinfectant [131-132]. However according to the EN 13697 bactericidal activity is defined as >4 $\log_{10}$ reduction of organisms attached to a surface under examination [133-134].

1.16.1. Tolerance to disinfectants

When cells in a mature biofilm come into contact with disinfectants, it is hypothesised that the cells deep within the biofilm matrix are protected by the outer layers of cells and extracellular polymeric matrix. As a result of this protection these cells only come into contact with sub-lethal doses of the chemicals therefore this may be responsible for both phenotypic and genotypic adaptation [105]. The stress adaptation by biofilm cells may also give rise to an increase in population of resistant or tolerant cells [76]. This may result in limited antibiotic options in the case of salmonellosis infection [135].

Braoudaki and Hilton highlighted this concern when they demonstrated that the use of disinfectants (benzalkonium chloride, chlorhexidine and triclosan) against *Salmonella enterica* may encourage cross resistance to antibiotics [136]. This research demonstrated that if *Salmonella* Virchow was exposed to sub-inhibitory concentrations of benzalkonium chloride it became relatively more resistant to 8/18 antibiotics tested in comparison to the parent strain [136]. Similarly Karatzas *et al.* found that when *S.* Typhimurium was exposed to sub-lethal doses of three disinfectants (oxidising compound blend, quaternary ammonium blend and a tar-acid

based disinfectant) the strains tested exhibited reduced susceptibility to antibiotics. This research reported that the adapted *S*. Typhimurium strains displayed a fourfold reduction is susceptibility to ciprofloxacin, chloramphenicol, tetracycline and ampicillin [128].

However other authors have suggested that the use of disinfectants such as triclosan may actually increase the *Salmonella* strains susceptibility to antibiotics such as ciprofloxacin [137]. As a result of contact with triclosan, Tabak *et al.* demonstrated that the *S*. Typhimurium biofilm cells had increased susceptibility to ciprofloxacin. The treatment involved sequential treatment of the biofilm (formed on the air-liquid interface) with 500µg ml^{-1} of triclosan for 30 minutes followed by 1 hour treatment with ciprofloxacin solutions (0-500 µg ml^{-1}) [137].

1.16.2. Methods for evaluating the efficacy of disinfectants against *Salmonella* biofilm

Disinfectant companies often market their products as active against *Salmonella* however these claims are often based on suspension test results on planktonic cells [134]. To investigate the efficacy of disinfectants in food processing and domestic environments, the methods applied should be as close to “real life” conditions as possible [130].

Suspension tests are usually performed using a microtitre plate based system where a panel of disinfectants at fixed concentrations are added to a known concentration of bacterial culture for a period of time after which the number of surviving cells is measured either by plate counts, optical density or staining [132, 138-139]. This method of testing culturable cells may have some relevance for studying the methods of cell entry and subsequent killing, however these tests are of limited value in assessing

activity in contaminated environments when cells are more frequently attached to surfaces and/or present in biofilm [140].

Other research groups have also created laboratory test environments to mimic the conditions found in food processing environments. Marin and colleagues seeded areas of a concrete floor with *Salmonella*, before allowing the development of a biofilm over the period of 3 days, after which the biofilm was disinfected with glutaraldehyde and formaldehyde, with contact time intervals between 1-60 minutes [141]. This recommended treatment was not sufficient to kill an established *Salmonella* biofilm in field conditions, irrespective of contact time, strain or serovar studied [141].

A more practical approach to testing the efficacy of disinfectants against bacterial biofilm formation, is to incorporate the materials that biofilm are regularly found on into the biofilm reactor. There are numerous reports on the use of stainless steel as a substrate for biofilm formation [101, 116, 142-148]. Unfortunately the wide variation in both procedures and evaluating of bactericidal activity results in difficulty in comparing the efficacy of disinfectants.

It is difficult under any circumstances to compare the results of tests of efficacy of disinfectants unless some of the parameters such as age of biofilm, flow method or enumeration remain constant throughout. In order to overcome these biases and obtain data on the biofilm reactors influence $\log_{10}$ reduction Buckingham-Meyer compared 5 different biofilm test methods. This work used identical surfaces (glass) repeating the same methods for biofilm removal, plate count enumeration and log reduction calculations (control coupon–treated coupon). The work evaluated biofilm

formation of two strains commonly associated with biofilm formation *Pseudomonas aeruginosa* and *Staphylococcus aureus* [117].

The methods applied by Buckingham-Meyer *et al.* varied quite significantly in relation to fluid shear and operation system (batch or continuous flow system). The CDC reactor (as previously described) and the drip flow reactor biofilm (coupons into reactor vessel with flow on inoculated medium passing through the reactor system) were used [117]. Other methods included a dehydrated biofilm system (coupons taken out of CDC reactor and dried in accordance to ASTM standards) and the dried surface method (heavy inoculum of test organism allowed dry onto the surface for 2 hours in accordance to the ASTM standards). There was a significant difference in density of biofilm formed on the surfaces and the reduction of viable cells after treatment with disinfectant agents, which is discussed further in chapters 2 and 4 of this thesis. The results suggested that incorporating a number of variables into a reactor model, such as a high shear environment, with continuous flow and mixing of nutrients may be essential for testing the efficacy of disinfectants against a biofilm, that may represent the most rigorous assessment of disinfectant activity against biofilm [117].

Differences between biofilm grown in different reactors have also been published elsewhere. Nailis *et al.* compared the phenotypic differences between *Candida albicans* biofilm when grown in the biofilm surface models (CDC biofilm reactor, microtitre based model) and biofilm animal models (subcutaneous rat model and reconstructed human epithelial model) [121]. Not only were there differences between the surface models and the biofilm animal models, but there were major differences between the CDC and the microtitre based model despite both growing the biofilm

of silicone disks [121]. This research demonstrated major differences in the level of gene expression responsible for virulence and adhesion factors.

1.16.3. Effect of age of biofilm on cell recovery

It is widely accepted that if the disinfectant does not penetrate and diffuse though the deeper layers of the biofilm, the biofilm may experience cell recovery and persist in the environment [56]. There is both limited and contradictory information on whether disinfectant effectiveness varies with biofilm stage of maturation [149].

Habimana and colleagues investigated persistent *Salmonella* strains (indistinguishable strains when examined using PFGE) which were repeatedly found in the same food processing plant [70]. Habimana *et al*. examined if the ability to survive stress conditions over 28 days, in comparison to other species found in the food processing environment, may have contributed to persistence of the *Salmonella* strains. The results indicate that *Salmonella* survival decreased linearly over time. These results indicated that the natural biota were able to survive in higher quantities than *Salmonella* in both the dry and humid conditions [70]. However, interesting results were highlighted when looking at the *S*. Agona as part of a mixed species biofilm. The cell density (measured in bio-volume of cells visualised using microscopy) of *S*. Agona increased when mixed with *Pseudomonas* species (3.2 fold increase) and *Staphylococcus* spp. (2.8 fold increase) in comparison to a mono-species culture of *S*. Agona. This suggests that *S*. Agona biofilm persistence on food processing environments may be supported by the presence of other organisms. It was also noted that *S*. Agona had a different morphology when grown in a mixed species biofilm and was found to grow under *Pantoea* spp. which formed a super-structures on top of the *S*. Agona cells. This could possibly

shield the *S*. Agona cells from direct contact with the disinfectant, resulting in lower levels of exposure to the disinfectant agents deep within the mature biofilm [70].

Ramesh *et al.* also investigated the persistence of *Salmonella* serovars regularly found in food processing environments (*S*. Typhimurium, *S*. Thompson, *S*. Berta, *S*. Hadar, and *S*. Johannesburg) found that quaternary ammonium compounds and an enzymatic biocide were not as effective against a 4-day *Salmonella* biofilm in comparison to a 3-day biofilm on stainless steel [150]. Similar findings were also reported by Korber *et al.* who found that trisodium phosphate was not as effective at killing *S*. Enteritidis when part of a 72 hour biofilm as a 48 hour biofilm [102]. Using a glass flow cell and electron microscopy Korber found that 75% of the *S*. Enteritidis biofilm cells remained attached at the base of the glass surface after treatment of the 48 hour biofilm with trisodium phosphate, however this increased to 91% when testing against the 72 hour biofilm [102].

The hypothesis that a mature biofilm subsequently leads to increased resistance or co-resistance to disinfectants and antimicrobial agents has also been substantiated elsewhere, particularly in dental research. Using bacteria derived from dental plaque. Shen and colleagues reported the thickness of a biofilm increased from 57µm (2 days) to 155 µm (3 weeks) during biofilm development, reaching a steady state of 190 µm after 6 weeks [151]. When testing the multispecies biofilm against chlorohexidine (CHX) they found that the proportion of killed bacteria in mature biofilms (>3 weeks) was lower than a biofilm formed over a shorter period of time (2 days, 1 and 2 weeks) after treatment with CHX. Shen *et al.* concluded that bacteria in nutrient limited biofilm formed over an extended period of time were more resistant to CHX than biofilm formed over a shorter duration of time [151]. In the study described by Shen *et al.*, a 3+ week

biofilm was considered mature based the structural development [151]. Lamfron *et al.* demonstrated that after 24 hours, a *Candida albicans* biofilm was more resistant to fluconazole than 2, 6 and 24 hour biofilm [152]. Xu *et al.* detailed how the biomass of a biofilm derived from sludge placed on a filter membrane increased over time durations of 6-, 12- , 24- hour was increased to 7.13, 17.49 and 24.43mg and that after treatment with adenosine triphosphate (ATP) synthesis inhibiter, the biomass was reduced by 68%, 80% and 34% respectively [153]. Fraud *et al.* showed that the activity of amine oxide (antimicrobial used for eradication of dental biofilm) reduced the bacterial count of *Streptococcus mutans* by over 5 log_{10} within contact with a 16 hour biofilm, however the efficacy of the antimicrobial agent was significantly reduced with mature biofilms (40-64 hour biofilm) with minimal activity observed [154]. Fraud *et al.* also acknowledged that the substratum may affect antimicrobial activity, to overcome this limitation all these experiments were carried out on hydroxyapatite disks to imitate the enamel of a tooth surface [154].

However other previously published material has suggested that age of biofilm does not contribute to a higher biofilm mass or increased resistance to disinfectant agents over time. Wong *et al.* found that the age of a *Salmonella* Typhimurium biofilm (3-, 5-, 7- day old biofilm) did not contribute to increased resistance to disinfectant using the MBEC system [149]. As discussed in chapter three, it may be possible that a smaller surface area available for biofilm development may have also contributed to no increased biofilm density or resistance to disinfectant agents over time.

Key Objectives of this study

The main objectives of this research were:

1. To investigate whether the strain of *S*. Agona SAGOXB.0066 that was responsible for a large food-borne outbreak differs in density of biofilm formation compared with other *S.* Agona strains and if any such differences are dependent on the biofilm substratum.

2. To evaluate if other serovars that ar*e* commonly associated with food-borne outbreaks, such as *S*. Enteritidis and *S*. Typhimurium, are similar to or different from S. Agona in relation to density of biofilm formed under the same conditions as the *S*. Agona investigated.

3. To examine the impact of time on density of biofilm formed.

4. To assess methods to eradicate *Salmonella* biofilm cells using disinfectants commonly associated with food processing areas.

Chapter 2

Assessing the density of a 48 hour *S. enterica* biofilm established using the CDC biofilm reactor

2. Abstract

Food-borne pathogens can attach to food contact surfaces. Bacterial attachment and survival in food processing environments may be assisted by the ability of the bacterial cells to form a biofilm. The biofilm may provide a protective layer of tightly attached cells and proteinaceous substances that shield the cells from harsh environments such as cleaning agents or dry conditions. In 2008, there was a large outbreak of *S*. Agona originating from a food processing plant. It was hypothesised that biofilm formation may have contributed to *S.* Agona persistence in the environment. This hypothesis has also been proposed in other food-borne outbreaks.

The research described in this chapter examines the density of biofilm formed by the *S*. Agona SAGOXB.0066 outbreak strains in comparison to other *S*. Agona strains. The density of biofilm formed by *S*. Agona strains was also compared to strains belonging to the *S*. Typhimurium and *S*. Enteritidis serovars. In order to assess the extent to which particular surfaces may differ with regard to their ability to support *Salmonella* biofilm, five surfaces comprising of glass, stainless steel, polycarbonate, tile and concrete were tested. All isolates form biofilm on all surfaces studied. Based on the enumeration of removed cells, the *S*. Agona strain associated with the outbreak did not result in more dense biofilm compared with other strains of *S*. Agona. However, in all instances *S*. Enteritidis biofilm density was lower than that of *S*. Agona and *S*. Typhimurium. The density of biofilm was dependent on substratum as biofilm density was greater on tile than concrete, polycarbonate, stainless steel and glass.

2.1. Introduction

It is estimated that up to 85-95% of salmonellosis cases are a result of food-borne infection [5, 155]. During the processing and handling of contaminated raw meat, work surfaces can become heavily colonized with

Salmonella enterica [29]. Once bacteria are introduced into an environment, the surfaces may provide a sufficient niche environment for biofilm development and persistence over long periods of time [140]. Vestby *et al.* found that *Salmonella* can survive in a food processing environment for up to ten years in a biofilm [56]. *Salmonella enterica* has been linked to contaminated equipment and processing lines [34, 36, 109, 156-157]. Previous authors have estimated that 29% of food-borne contamination arises from cross contamination during raw meat preparation [158-159]. These findings suggest that contaminated food contact surfaces may provide *Salmonella* with an entry into the food chain. Due to this association, food contact surfaces have been the subject of several studies including adherence to surfaces [100, 108, 160], survival and transfer from surfaces [30, 150], biofilm studies [56, 71, 98, 161] and environmental monitoring studies [141, 162-163].

2.1.1. Surface differences

Despite research into biofilm formation on food contact surfaces there have been limited comparisons of multiple contact surfaces within the same model. The surface or substrata to which the cells attach to and develop a biofilm on may be important factors influencing the bacterial attachment, growth, morphology and ability to resist disinfectants.

Chia *et al.* demonstrated that the surface properties teflon, stainless steel, glass, rubber and polyurethane may influence the level of *S. enterica* attachment [100]. Chia and colleagues found that the *Salmonella* attached to teflon in higher numbers than the other 4 materials. There was no significant difference between glass and stainless steel or rubber and polyurethane ($p<0.05$). The mean adherence for all of the 25 strains was highest for teflon followed by steel, glass, rubber and polyurethane [100]. Although surface properties influence the level of attachment, Chia *et al.* concluded that the degree of variability between strains observed

indicated that there may also be other components responsible for variation and that attachment may be strain dependent. Previous authors have suggested the surfaces properties can influence the amount of bacterial attachment and subsequent biofilm formation [103].

Joseph *et al.* found that biofilm development was dependent on substratum with two *Salmonella* strains (*S*. Weltevreden and un-typable *Salmonella* denoted as “FCM40”) [71]. *Salmonella* formed the most dense biofilm on plastic followed by cement and stainless steel respectively. Joseph *et al*. also found that inactivation of *S*. Weltevreden biofilm cells with hypochlorite (no cells detected) was more rapidly achieved on cement and steel than plastic (15 min vs. 20 minutes). Similar results were displayed for the FCM40 strain, with a slightly higher exposure time necessary (20/25minutes) [71]. However it is difficult to define the extent to which one can generalise from the two strains studied.

Previous research has demonstrated that the level of bacterial attachment to stainless steel can be subject to the surface roughness and finish of the steel [103, 164]. Korber *et al.* demonstrate that surface roughness can influence *S.* Enteritidis susceptibility to disinfectants [102]. The presence of crevices in a glass flow cell appeared to protect the *S*. Enteritidis from treatment with the disinfectant trisodium phosphate (TSP). The researchers found almost 100% of biofilm cells were killed from the model containing a smooth substratum, whereas up to 83% of cells on a rough surface remained viable after contact with the same concentration of TSP. The research also indicated the size of the crevices influenced the biofilm with smaller crevices supporting bacterial survival and the thickness of the biofilm after contact with the disinfectant [102].

Stocki *et al.* also highlight that the extent of surface area (related to layers of plastic material) may also influence *S*. Enteritidis biofilm development on surfaces. Results indicated that the type of smooth materials such as vinyl

allowed increased removal after disinfection in comparison to the woven materials with a higher surface area [67]. The aforementioned projects each examined different finishes of a single surface material of steel [103], glass [102] and synthesised plastic materials [67].

2.1.2. Strains/ Serovars

Rodrigues *et al.* confirmed that *S*. Enteritidis strains were able to form a biofilm on kitchen surfaces such as silestone (impregnated with triclosan), granite, marble and steel [98]. Through the use of staining and viable plate counts Rodrigues *et al.* found that four of the five strains tested were more adherent to marble than any other surface. Rodrigues also suggested that there may be strain variability as in most instances the food isolate and control isolate formed more dense biofilm than clinical isolates in a 6 well microtitre plate model. The researchers incorporated multiple surfaces into the biofilm model, however, all of the strains tested were *S.* Enteritidis. It may have been of value to also investigate whether there was any inter-serovar variation in the density of biofilm formed on the surfaces which has been suggested elsewhere [56, 76, 165]**.** The authors did not incorporate any sheer stress into the biofilm model design used. This may be significant as the introduction of sheer stress has been identified as an important variable in biofilm formation [116-117, 166].

2.1.3. Origin of strains

The source of origin of strains may have an impact on the ability of the organisms to form a biofilm. Castelijn and colleagues reported that the density of biofilm formed in altered concentrations of nutrient-rich typtone soy broth (TSB) was related to the origin or source of the 51 *S.* Typhimurium strains examined [32]. Both strains, traced to outbreaks and to contaminated retail products resulted in dense biofilm formation at 25°C and 37°C in the nutrient-rich and diluted media. However the isolates

taken from industrial settings only displayed dense biofilm formation in nutrient-limited environments (1/20 diluted media). This was interpreted as suggesting that most of the clinical and retail product associated isolates were adapted to nutrient-rich environments and body temperature [32]. The work described by Castelijn *et al.* suggests that the strains isolated from the industrial environments may form a less dense biofilm due to the environmental conditions the strains were isolated from, possibly though adaptation to a niche environment. However, Castelijn *et al.* did not give any indication of the duration the environmental isolates were present in the environment before isolation for biofilm studies [32]. Therefore, it is possible that the strains examined may be planktonic cells recently introduced to the environment from food sources. As a result, these strains may not be any different to the food related counterparts.

Castelijn *et al.* indicated that *S*. Typhimurium strains LT2 (characterised with over expression of RpoS) and L2 (a variant of LT2 lacking expression of RpoS) had similar biofilm density levels in the microtitre plate method. The LT2 strain has been considered by others as a dense biofilm former due to the expression of the stationary-phase sigma factor σS (RpoS) [167-168]. On that basis, one might anticipate that the L2 variant lacking this expression should be a less dense biofilm former and previous studies have in fact reported notable differences between LT2 and L2 variants [169-170]. It is possible that expression of *rpo*S may be less critical to biofilm formation in the model used by Castelijn *et al*. for some reason however, the authors do not address this difference between their findings and previous reports.

Previous authors have reported that strains recovered from food processing environments repeatedly over an extended period of time, were more dense biofilm formers than reference strains from the *S*. Montevideo and *S*. Agona serovars [56]. Vestby *et al.* also reported that

although *S*. Senftenberg was the serovar most frequently isolated from the Norwegian food processing plants investigated, it persisted in the environment for a shorter period of time than the other serovars studied. Therefore the authors concluded that the frequent isolation of *S*. Senftenberg may be due to presence in raw material and not relating to its ability to form a biofilm [56]. This research provided by Vestby *et al.* suggests that the *S*. Senftenberg recovered from the surface may have been in a planktonic form on the surface while the other strains recovered were more representative of dense biofilm formers due to repeated isolation of specific strains from the environment [56].

Rodrigues *et al.* suggested that *S*. Enteritidis biofilm formation may be strain dependent but that the volume of biofilm formed may also be surface or substratum dependent. Rodrigues *et al.* reported that isolates recovered from food and reference isolates formed a less dense biofilm than clinical isolates of *S*. Enteritidis using the microtitre plate based system [98]. These findings correspond with Castelijn's hypothesis that clinical isolates may have a higher propensity to form a more dense biofilm than strains associated with food or food processing environments [32]. Other studies were unable to confirm a relationship between source or origin of isolates and ability to form a biofilm/density of biofilm formed [96, 111]. However given the small test sample size, such as a total of five *S*. Enteritidis isolates used by Rodrigues *et al.*[98] incorporating only 1 food isolate it is difficult to determine if this trend would be still be evident with a larger sample size. As a result, it may be more accurate to conclude that the biofilm density is more likely to be strain dependent than source dependent.

2.1.4. CDC Biofilm Reactor

The CDC Biofilm Reactor (CBR) is a one litre vessel with an effluent spout positioned to hold approximately 350ml of media once assembled. Continuous mixing of the reactors bulk fluid is provided by a magnetised baffled stir bar, which is controlled by a digital stir plate to determine the rates per minute (rpm). The top of the reactor consists of polyethylene top with three inlet ports (media, gas exchange and inoculation port) the top also supports eight independent rods. Each rod accommodates three removable coupons (biofilm growth surfaces) for a total of 24 sampling opportunities. The reactor is connected to two carboys (large autoclavable re-usable plastic bottles). The fresh media carboy and waste media carboy are connected to the CBR using silicone tubing. The culture was circulated into the reactor via sterile tubing which is fed through a peristaltic pump which controls the flow rate through the reactor. An advantage of the CBR is that the mixing and feed rate are optimum (the inflow rate is identical to the outflow rate) which is designed to flush out planktonic cells whilst only leaving the sessile cells in the reactor. This method also ensures that the conditions are as near as possible to identical in all locations within the reactor [171]. The inlet spout is connected to a glass flow-breaker which prevents contamination from the inoculated media from entering the clean media if there is a reflux in the piping. The CBR reactor is set-up to run within the confined area of a fume hood, the tubing is secured in position (above the inlet spout) to the wall using heavy duty duct tape. All components of the CBR are autoclaved before and after use. Images of the CBR and equipment are provided in figures 2.1 and 2.2 on page 63.

2.1.5. Advantages and disadvantages of the CBR

The main advantage of the CBR is the ability to examine up to 24 individual coupons simultaneously. This means that research such as investigating biofilm density or protein development at different time points during biofilm development and on different substrata can be assessed simultaneously under uniform conditions using one reactor [121]. It also

facilitates testing of different disinfectants at varied concentrations on coupons that carry biofilm which has developed under uniform conditions [117]. Previous authors have also demonstrated that the CBR results in a more dense biofilm and a smaller log_{10} reduction after contact with disinfectant agents than other methods such as drip flow reactor or the current standard of assessing the efficacy of a disinfectant against a microbial suspension attached to a surface (EN13697:2001) [117]. The European Committee for Standardisation (CEN-Comité Européen de Normalisation) EN13697:2001 and other current standards are described in greater detail in chapter 4. It is possible that the larger surface area provided by the coupons in the CBR in comparison to other biofilm reactor models such as the microtitre plate based system may be responsible for a more dense biofilm formed on the surface over the allocated period of time [165]. The use of multiple surfaces and provision of a larger surface area exposed to nutrient flow and mixing or shear stress may also be more representative of the conditions found in food processing.

However there are also a number of disadvantages to the CBR. Firstly, the CBR is designed for batch-flow and continuous flow provision of sterile media to optimize biofilm development. Therefore, given the size of the reactor and the flow rates chosen (0.5-11ml $minute^{-1}$) a large quantity of media is required to achieve the biofilm development over the continuous flow period. For a flow rate of 1ml $minute^{-1}$ over 7 days over 14 litres of fresh media is required. The process of replenishing supply of sterile media also allows opportunity for contamination unless strict aseptic techniques are applied. Therefore the CBR is costly and time consuming in comparison to other methods that can use a 10/100 fold less quantity of test chemical per experiment such as the MBEC method described in chapter 1.

Performing disinfectant assays using the CBR requires more handling of the substrata and materials such as sterilized tweezers, glass containers, pipettes, syringes and plates whereas the use of a microtitre plate method

can be performed without the labour intensive process within a closed sterile environment. The handling of the surfaces for enumeration of viable cells also allows an opportunity for contamination if aseptic techniques are not maintained.

2.1.6. Reproducibility of biofilm studies using the CBR

In addition to examining biofilm density on multiple surfaces and using multiple strains it is also important to investigate the limits of intra-laboratory reproducibility derived from repeated experiments. Reproducibility is frequently represented through the use of the standard deviation (SD). In similar research using the CBR and polycarbonate as a biofilm substrata Goeres *et al.* found the SD of the mean log_{10} density to be 0·30 for *Klebsiella pneumoniae*, 0·22 for *Streptococcus pneumoniae* and 0·17 for *Pseudomonas aeruginosa* [72]. Goeres and colleagues investigated intra-laboratory reproducibility (also referred to as repeatability) and the impact of altering the conditions such as flow rate, media concentration and stir rate. In total 21 experiments were performed and the results were assessed on triplicate coupons taken at random from each run. Replicate experiments were undertaken to investigate the reproducibility under each alternative set of conditions. Goeres found that overall that altering the conditions slightly such as 2°C temperature change or 10% reduction in concentration of media did not significantly reduce the density of biofilm formed on the surface [72].

Buckingham-Meyer *et al.* also compared the SD of measures of *S. pneumoniae* and *P. aeruginosa* biofilm density attached to glass coupons using the CBR model and four other biofilm models [117]. All experiments repeated in duplicate with two glass coupons used in each experiment for all models (*n*=4). The other models included a drip-flow reactor model (inoculum flowed over the coupons using syringe device), a static biofilm model (inoculum infused in solid media in contact with the surface over

time) and a dried biofilm (cell suspension and organic substances were dried onto the surface to simulate organic soil). The comparative work also incorporated a dehydrated biofilm model where the coupons were released from the CBR and dried in a petri-dish over a 2 hour period. The SD for the mean log_{10} density of cells on the two glass coupons was 0.211 and 0.224 for *P. aeruginosa* and *S. pneumoniae* biofilm respectively [117]. When examining *P. aeruginosa* biofilm density, the CBR model had a lower SD than the drip-flow (SD of 0.788), the dehydrated biofilm (0.548) and dried surface biofilm (0.435), but a slightly higher SD than the static biofilm (0.208). The *S. pneumoniae* biofilm density assessed in the CBR had a lower SD than the drip flow (SD of 0.586), the static biofilm (0.330) and the dehydrated biofilm (0.322). The dried surface biofilm had a lower SD (0.157) [117].

Donlan *et al.* described the assessment of *S. pneumoniae* biofilm density *in situ* and in real time using the CBR model [124]. The biofilm was developed on germanium coupons and biofilm cell density was measured using the plate count technique after 21, 45, 69, 141 and 189 hours development. The mean log_{10} density and SD was calculated from 3 replicate coupons, with 2 coupons used to calculate the SD of the 141 hours incubation time. There was no indication if the replicate coupons were taken from the single or multiple runs using the CBR. The SD for biofilm density was between 0.06-0.34 for the five time points [124]. Hadi *et al.* used X-ray diffraction, spectroscopy and SEM to examine the density of *P. aeruginosa* biofilm formed on plastic (polytetrafluoroethylene) coupons using the CBR [123]. Hadi *et al.* reported an SD of 0.655 through crystal violet staining and measurement of the biofilm through a commercial assay used to quantify the volume of proteins remaining on the surface. The SD was calculated on 9 coupons from each run, performed in triplicate (n=27) [123].

To summarise, previous authors using the CBR have investigated intra-laboratory reproducibility with a varied number of repeated samples, methods for enumeration and organisms under examination. The extent of variation described in the aforementioned research was standard deviations ranging from 0.06 [117] to 0.655 [123]. In general, the data suggest that intra-laboratory variation of biofilm density with the CBR is less than that observed with some other systems. It is important to note that only one surface material was chosen for each assessment described above.

2.2. Examining biofilm density and reproducibility using other biofilm reactor models

In research similar to that described in this thesis, Joseph *et al.* investigated the biofilm density of 3 *Salmonella enterica* serovars in duplicate on concrete (referred to as cement), plastic (high density polyethylene) and stainless steel using a microtitre plate system[71]. However the work did not report the SD or provide any indication if replicate experiments were performed [71]. The work performed by Joseph *et al.* is widely used as a reference for evidence of biofilm formation on contact surfaces and resistance to sanitizers despite any indication of reproducibility or robustness of the method used.

Rodrigues *et al.* investigated total biofilm developed over 48 hours (measured by crystal violet staining) and viable cells using the plate count technique [98]. Biofilm density of five *S.* Enteritidis strains attached to granite, marble, stainless steel and two types of silestone impregnated with the antimicrobial agent triclosan were assessed. The results of the research indicate that the SD varied depending on surface and strain with the mean ranging from 6-7 $\log_{10}$ CFU/cm^2 for most surfaces while the SD

range of 0.05-0.12 on granite, 0.05-0.22 on marble, 0.05-0.21 on stainless steel, 0.10-0.41 on white silestone and between 0.01-0.19 on beige silestone [98]. The SD values were based on four repeated experiments with three replicates used on each occasion (*n*=12). The low SD values reported in the text may indicate the level of stability when using a closed batch-flow model where each test material is examined separately (no mixing of nutrients or planktonic cells in contact with multiple surfaces) [98]. Moreover, the limited number of variables such as flow of nutrients, shear stress and position within a multi-coupon reactor system (robustness of method) may have contributed to the low SD values gained in the research.

Stocki *et al.* examined biofilm formation of *S*. Enteritidis and *S*. Typhimurium on plastic materials (vinyl, nylon, hemp plastic, farmers belt, plastic) commonly used for manufacturing conveyor belts in egg processing facilities [67]. Stocki and colleagues calculated the mean $\log_{10}$ density of cells of the five belt types by enumerating the $\log_{10}$ CFU/cm^{-2} of cells recovered from a set of 5 belt pieces stored in 3 petri dishes (*n*=15 for each belt type).The surfaces were incubated at 28°C for a total of 14 days to form a biofilm, consisting of 7 days incubation pre- and 7 days post-treatment with disinfectant. Despite the repeated measures and extensive use of replicates, Stocki *et al.* found large variation in measurement which was still evident post log transformation of the data. The large variation between repeated measurements was highlighted through the use of interquartile range for non parametric data which ranged between 0-2.5 $\log_{10}$ CFU/1cm^2 depending on material and strain tested [67].

2.3. Methods for examining biofilm density and substrata

2.3.1. Atomic Force Microscopy (AFM)

Atomic force microscopy uses a scanning cantilever or probe that taps the surface of a specimen on contact. The probe then gently scans the surface and based on tapping of the surface encountered. A laser beam is aimed at the outer area of the probe. The electrons emitted from the laser are deflected back to a photo-detector which deciphers the distance of the probe from the surface at each scanned point to measure surface roughness of the overall sample [172] .

2.3.2. Scanning Electron Microscopy (SEM)

SEM is a widely used method for examining biofilm on surfaces [114, 122, 173-174]. SEM has previously been used and standardised by Williams and colleagues to confirm biofilm growth of *Staphylococcus epidermidis* established using the CBR [174]. Gilmore *et al.* also used SEM analysis to confirm dense biofilm formation by *Proteus mirabilis* using the CBR [122]. The basic principle of SEM is that a beam of electrons are emitted from an electrode gun and accelerated at high voltage (e.g. 20 kiloVolts - kV) onto a specimen. Once the beam of electrons hits the specimen, the electrons rebound off the surface and are collected through the secondary electron imaging (SEI) collector [175]. The SEI collector deciphers the electrons collected and coverts them to digital analogs or signals. The signals are then converted to grey-scale pixilation using computer programming [175].

In order to optimize conditions for SEM visualization, the specimens are fixed (primary and secondary fixatives) which preserve the cellular details of the biofilm [174]. After fixation the specimens are dehydrated in a series of ethanol to remove excess water from the sample [175]. Any water remaining may destroy the sample if overheated in the SEM chamber. The ethanol series is followed by the addition of a critical point dryer to eliminate surface tension from liquids on the surface of the specimen. Hexamethyldisilizane (HMDS) is now recognised as a safer option for

critical point drying due to safety concerns of critical point drying chambers (due to over heating or excessive pressure). After drying the specimens are coated in a thin layer of gold (or other conductive materials). This allows the specimen to emit the electrons when they hit off the surface and therefore be collected by the detector. The use of gold coating has been demonstrated to produce sharper images of biofilm cells compared with specimens that are not coated. The specimens are also mounted onto carbon double sided stickers and metal stubs to prevent movement during the process (movement could be caused by the vacuum created) the carbon and metal also provide conductivity.

2.3.3. Other methods for examining and enumerating biofilm cells

A number of authors have reported on the use staining methods such as LIVE⁄DEAD® biofilm viability kits with confocal laser scanning microscopy (CLSM) and computer programmes to measure biofilm density relative to the surface area of the biofilm substrata [123, 176-178]. The LIVE/DEAD® stain contains the nucleic acid binding dyes SYTO® 9 green and propidium iodide. When used in combination, the SYTO® 9 green attaches to all cells while the propidium iodide penetrates disrupted cell membranes (non-viable cells). Hadi *et al.* described the use of LIVE/DEAD® stain to examine the percentage coverage *P. aeruginosa* biofilm formed on polytetrafluoroethylene (plastic) coupons developed using the CBR [123]. The use of LIVE⁄DEAD® stain [176-177] and SYTO® 9 green [178] to investigate biofilm formation using hydroxyapatite (dental ceramic) disks, glass flow-cells and microtitre plate models has also been reported elsewhere.

However due to the surface properties of the coupons used in this thesis, in particular polycarbonate, concrete and tile, there was a high level of autofluorescence, when examined using confocal laser scanning microscopy. Autofluorescence of the surface may result in inaccurate measurement of the biofilm or lack of discrimination between the surface

and the biofilm formed therefore comparison of biofilm density across five surfaces would be difficult. Moreover, despite the introduction of computer based programmes for measuring biofilm volume based on confocal images limitations such as miscalculation of density still remain unresolved [179]. Therefore CLSM and SEM still remain largely qualitative methods for assessing biofilm density.

Grid counting of SEM images involves dividing the surface area into small grids within which the cells are then counted to allow for an estimate of the total population. This method allows enumeration of all cells attached to a surface, therefore eliminating the need for sonication and culturing the bacteria and plate counting. The main disincentive to using grid counting is that it does not give any indication of the volume of live cells contained in the total biomass. Moreover, based on the dense structure of the biofilm cells and the presence of an EPS outer layer, it is not always possible to differentiate single cells for enumeration as depicted in the SEM images provided in this thesis.

In light of the advantages and disadvantages associated with the use of microscopy, enumeration via the plate count method remains the most frequently used method for biofilm measurement [68]. The plate count method involves removal of the biofilm (usually performed via scraping, sonicating or vortexing) followed by re-suspension and disaggregation of the biofilm in a liquid before spreading onto solid media and cultured over night at optimum temperature.

2.3.4. Sonication as a method of removal for enumeration

Ultrasonication (sonication) was chosen as an appropriate method for biofilm removal in this thesis. Under the optimum conditions, sonication should remove biofilm cells from a surface without disrupting or damaging the cells. The use of sonication has previously been demonstrated to be an

effective method for removal of biofilm cells from surfaces [72, 114, 118, 120, 122, 180-182]. The surfaces under examination are placed in sealed containers of sterile liquid. During the procedure the ultrasonic vibration leads to the formation of small bubbles in the liquid containing the surfaces. As a result the bubbles interact and hit off the surface resulting in the biofilm becoming dislodged from the surface without disrupting the integrity of the biofilm cells. The placement of the water-bath within the sonication unit allows the frequency of the sound to travel at a faster pace. However the challenge of sonication is to optimize removal of the biofilm matrix while simultaneously preventing cell damage [105]. The sonication water-bath controls the intensity of ultrasonic waves emitted per second (kiloHetz - kHz) by increasing the pitch and the frequency of the resonance sound. The degas function on the water-bath reduces the volume of gas naturally contained in the liquid solution before the process begins. The temperature of the water-bath can also be optimized by use of the heating block built into the bath. Room temperature can be maintained using a thermometer positioned in the bath with the addition of warm water or ice to alter the temperature.

Monson and colleagues highlighted that that in order to sufficiently remove biofilm cells from a surface whilst detecting and preventing cell damage quality checks must be performed to optimize conditions for the organism and surfaces tested [181]. Quality checks can be verified quantitatively by performing viable plate counts on the organism when in planktonic form (normal bacterial suspension) and also by comparing the sonication method for removal of bacteria to other methods. Other methods used for biofilm removal for cell enumeration include scraping the surface to dislodge the biofilm matrix [98] or swabbing [71]. However all removal methods should also be verified preferable through microscopy to confirm level of removal from the surface [183].

2.4. Statistical Analysis

Statistical analysis was performed with IBM SPSS version 20 (Statistical Package for Social Sciences co-branded by International Business Machines corporation). The data was first assessed to determine if the distribution was parametric or non-parametric. As a result, the subsequent tests performed were based on this criterion.

Parametric data is frequently referred to as normal data as it infers the data follows a normal (Gaussian) distribution [184]. Normal distribution is based on the assumption that 95% of all the data points fall within 1.96 of the standard deviation (SD) of the mean. Parametric tests include *t*-tests (comparing 1/2 groups) and ANOVA (analysis of variance) which can analyse the variance of multiple groups simultaneously. Parametric data are considered more powerful as all the data fits within the 1.96±SD. Non-parametric data do not fit any ordered distribution. Tests for non-parametric data are also referred to as distribution-free methods. Methods of analyzing non-parametric data include the Mann-Whitney U test (2 comparisons) and the Kolmogorov-Smirov Z test for multiple comparisons [184].

Comparing groups to evaluate if there are differences between them is usually performed by finding a p value. The null hypothesis (H_0) is that the result is likely to have occurred by chance and that given a larger set of values the same results would not occur. The alternative hypothesis (H_A) states that the result is unlikely to have arisen by chance and that given a larger dataset this result would still be statistically significant. For example if a null hypothesis was set that there was no difference between series of observations of A and a series of observation of B and the alternative hypothesis would be there was a difference between them. If the resulting p values from either parametric or non parametric tests was <0.05, this is

generally accepted by convention as indicating that the null hypothesis can be rejected, meaning that there is a statistically significant difference between observations for A and the observations for B.

Type I errors (the false appearance of apparent significance) and type II errors (the failure to detect an actual significant difference) can occur. Therefore it is necessary to exercise caution when interpreting *p* values. Researchers should be aware at all times that the use of a *p* value only suggests the presence or absence of a statistically significant difference. Moreover, the determination of *p* values on multiple groups or comparisons may increases the likelihood to type 1 errors. It is reasonable to think that if multiple tests are performed it becomes more likely that a value of <0.05 will occur by chance. In this case, the researcher may wish to correct for this possible error. The use of Bonferroni correction is frequently used for this assessment where the significance threshold would be divided by the number of comparisons made [67, 108]. For example, if 20 comparisons were made in a test, based on significance indicated when $p=0.05$, the significance would only be reached if $p=0.0025$. However there are limitations also regarding the application of these corrections. Because all statistical methods and corrections have limitations for this thesis project it was considered appropriate to present uncorrected *p* values as a broad indication of likely significance of difference but with the qualifications on interpretation outlined.

Table 2.1: Strain characteristics of all 13 strains.

Strain	Serovar	Strain Characteristics			Origin
		Antigenic Structure	Phage type	PFGE Profile†	
27853	*P*. aeruginosa	n/a	n/a	n/a	ATCC Control Strain
S08-0601	*S*. Agona	4,12:f,g,s	PT39[1]	SAGOXB.0066[3]	Human faecal sample
S09-0494	*S*. Agona	4,12:f,g,s		SAGOXB.0066[3]	Environmental swab from food plant
S09-0046	*S*. Agona	4,12:f,g,s		1 Band variant to SAGOXB.0066	Drain swab
S09-0371	*S*. Agona	4,12:f,g,s		IE09019-14[4]	Human faecal sample
S09-0479	*S*. Agona	4,12:f,g,s	PT3[1]	IE09021-07[5]	Sesame seed sample
SL483	*S*. Agona	4,12,f,g,s		25.H.03[6]	Control strain Accession No:CP001138
S09-0419	*S*. Typhimurium	4,5,12:i:-	DT193[2]	STYMXB.0131[7]	Porcine carcass swab
S08-0408	*S*. Typhimurium	4,5,12:i:2	DT193[2]	STYMXB.0131[7]	Human faecal sample
SL1344	*S*. Typhimurium		n/a	n/a	Control strain Accession No:FQ312003
PB449/LT2	*S*. Typhimurium		n/a	n/a	Control Strain LT2
S09-0717	*S*. Enteritidis	9,12:g,m	PT14b [1]	n/a	Human faecal sample
S09-0004	*S*. Enteritidis	9,12:g,m	PT4 [1]	n/a	Imported Brazilian chicken

Table 2.1 describes the characteristics of all 13 strains examined in this thesis, the strain collection incorporated 1 *Pseudomonas aeruginosa*, 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *S.* Typhimurium and 2 *S*. Enteritidis strains. [1] Denotes the phage type (PT) of the strain. [2] Denotes the definitive phage type (DT) of the strain. [3] Denotes the profile of the strain based on the Pulse Field Gel Electrophoresis (PFGE) assessment. [4-5] Denotes the PFGE reference number for the *S*. Agona strains not related to the SAGOXB.0066 strains. [6] PFGE profile produced *in silico* by Mark Achtman Group [185]. [7] Denotes the PFGE profile of the two *S*. Typhimurium strains examined in this thesis. See Appendix 3 for PFGE images supplied by the NSSLRL.

2.5. Methods

2.5.1. 48 hour biofilm development method

I. All experiments were performed in accordance with the current good laboratory practices and health and safety requirements outlined by National University of Ireland, Galway.

II. The test organisms were stored at -20°C on glycerol protect beads between experiments.

III. Before each experiment the test strain was defrosted on the bench and streaked on Tryptic Soy Agar (TSA) and Xylose Lysine Deoxychocolate (XLD) plates which were incubated for 24 hours at 37°C.Isolated colonies were picked off the TSA plate and made to a 0.5 McFarland suspension (~$1.5x10^8$ CFU/ml) in sterile saline.

IV. 1ml of the suspension was added to 9ml of sterile Tryptic Soy Broth (TSB) and incubated in shaking incubator for 24 hours at 37°C.

V. Twenty coupons (four of each of the five coupon types - glass, steel, polycarbonate, tile and concrete) were cleaned for use in each reactor run.

VI. Coupons were sonicated at room temperature for 30 minutes in a detergent solution (2% Micro-90).

VII. Coupons were then rinsed and sonicated for an additional 30 minutes in reagent-grade water.

VIII. Using a 24-well plate the coupons were immersed in 1ml of 100% ethanol for 2 hours, ethanol was chosen as opposed to 2M HCl as this may chemical may result in degradation of surfaces i.e. rusting of stainless steel.

IX. The coupons were visually inspected to ensure there was no degradation prior to use.

X. The reactor was cleaned with micro-90 and ethanol prior to use.

XI. The coupons were fixed into the reactor rods using a sterile forceps and set-screw, with the test area of the coupon (face) was placed inward exposed to the sheer force provided by the baffler rotator (see Figure 2.1).

XII. The gas port and media inlets and waste port were sealed and the device was autoclaved prior to every use.

XIII. Any dislodged coupons were aseptically re-inserted into holders and the device was filled with 350ml of TSB (30g/L).

XIV. 10 litres of diluted TSB (10g/L) were autoclaved in the carboy.

XV. All tubing was connected to fit through carboy cap, fluid break and reactor attachment were assembled and autoclaved.

XVI. The reactor system was assembled (see figure 2.2).

XVII. The reactors were placed on the magnetic stirrers which rotate at 160rpm, maintained at room temperature.

XVIII. The flow rate of 1ml/minutes was confirmed after each assembly before inoculating reactor.

XIX. The reactors were inoculated with overnight suspension of the test strain.

XX. The density of the inoculum (CFU/ml) was confirmed using spread plate onto TSA agar.

XXI. The reactor was run on batch phase for 24 hours.

XXII. After 24 hours the reactor system was operated under continuous flow phase for an additional 24 hours.

XXIII. The reactor system was disassembled.

XXIV. All tubing and carboys were covered and autoclaved immediately.

XXV. Using a sterile forceps and set screw the coupons were extracted from the reactor and placed within the confines of a bio assay tray in the safely cabinet.

XXVI. Using a sterile syringe the coupons were washed with 20ml sterile H_20 to remove any planktonic cells from the surface and placed in the wells of a sterile 24-well plate.

XXVII. During each run care was taken to ensure the face of the coupon directly exposed to the shear stress of the reactor was facing upwards during the handling and sonication of coupons (particularly important for steel, polycarbonate and glass as both sides are identical).

XXVIII. Three sets of coupons (a set containing one of each of the 5 coupon types) were placed in individual capped universals with 10ml of sterile Phosphate Buffered Saline (PBS) and sonicated for 7 minutes at 20 kHz at room temperature

XXIX. The fourth set of coupons were placed in a 24 well microtiter plate and used for SEM analysis (see section 2.1.1).

XXX. After sonication the coupons were aseptically removed from the universals using a sterile forceps.

XXXI. Three sets of coupons were vortexed for 30 seconds (within the capped universals) to break down biofilm clumps and serial dilutions were made of the PBS solution.

XXXII. Serial dilutions of $1x\ 10^{-4}$ and $1x10^{-5}$ were made.

XXXIII. 100µl of the serial dilution was placed onto TSA and spread evenly over the surface of the plate using a spreader.

XXXIV. The TSA plates were incubated inverted for 24 hours at 37°C.

XXXV. A suspension from the reactor was plated onto XLD and CLED following serial dilution to confirm the broth only contained the test organism.

XXXVI. The number of colonies formed on each plate (CFU/plate) was counted after 24 hour incubation.

XXXVII. The CFU/plate were converted to Mean Log_{10} Density values for statistical analysis using the formula adapted by Goeres *et al*. 2005 [72].

XXXVIII. Mean Log_{10} density (CFU/coupon) = log_{10} (mean CFU per plate) + log_{10} (vol. sonicated into) + log_{10} (dilution factor) - log_{10} (vol. plated) - log_{10} (surface area of the entire coupon).

2.5.2. Scanning Electron Microscopy

2.5.2.1. Primary and Secondary

I. The coupons were immersed in primary fixative overnight (12 hours).

II. Primary fixative consisted of 5% Gluteraldehyde: 10% Paraformaldehyde solution in 0.2 Mole Cacodylate: hydrochloric acid buffer.

III. The primary fixative was taken out of the wells using a sterile pipette and replaced with 1ml of freshly prepared secondary fixative for 2-3 hours.

IV. Secondary fixative consisted of 2% Osmium Tetroxide 0.2M Cacodylate: Hydrochloric acid buffer.

V. The coupons were then dehydrated through a series of graded alcohols 30%, 50%, 70% and 100%.

VI. All wells were filled and emptied individually to ensure the samples did not dry out in between grades.

VII. The coupons were then placed on the reverse of the 24-well plate and using a pipette 100µl of drying agent HMDS (hexamethyl disilizane) was placed on the exposed face of each coupon.

VIII. All coupons were air-dried in a fumehood for 2 hours.

IX. The samples were then fixed on to metal stubs using carbon adhesive tabs.

X. The tile coupons were fixed with silver paint as the carbon tape would not adhere to the reverse of the tile coupon.

2.5.2.2. Gold Coating Samples

I. Two sets of samples were used for SEM analysis. Set 1: Biofilm intact (not sonicated) and set 2: Biofilm removed (sonicated for enumeration).

II. The samples were mounted onto metal stubs and fixed in place using double sided carbon coated tape.

III. The samples were placed in the gold sputter chamber into the assigned position. Identifying labels were placed on the reverse of the stubs.

IV. The inside of the chamber was cleaned with lint free paper and lid was closed firmly to ensure that there was no air allowed in (the chamber will not close and create an air tight vacuum in the presence of dust particles).

V. A vacuum was created by turning on gas cylinder the dial was adjusted (on the gas outlet) to alter the pressure and allow entry into the chamber and the sputter chamber was started

VI. The dial on the gold sputter was assessed for adjustment (if the dial was not moving the cleaning process may have to be repeated).

VII. After completion the dial readjusted to 0.7mBar and the pressure was turned off automatically. The vacuum was released in the chamber to open the lid.

2.5.2.3. SEM Analysis

I. The exchange position on manual levers of the SEM microscope was adjusted before opening.

II. >20mm - X axis (left-right), >20mm - Y axis (up-down), >Ex-Ex (exchange size of stage).

III. The vacuum within the chamber was released to open the chamber and load specimens.

IV. The coupon (now fixed, gold coated and set on metal pin stub) were height adjusted using the metal height ruler.

V. The vacuum is started under the appropriate conditions:

  - Conditions: Vacuum created with 25.0 kilo Volts (kV) with high resolution, a beam of 50 and working distance variable depending on the specimen under examination (used to bring the specimen into focus).

VI. If the kV is adjusted, the filament is re-saturated (usually after entry of new specimen into vacuum)

VII. >Help >Gun Alignment >execute >Help >Filament >Re-saturate the filament.

VIII. Low magnification was used to get the image in focus. (“TV2 Settings” to scan item) once the object was in view “TV3 setting” was used for finer focus.

IX. Once the conditions were set and the microorganisms were visualised and in focus the images were taken and saved.

2.5.3. AFM Analysis

I. AFM analysis was performed to determine the roughness of each of the five surfaces.

II. The 5 surfaces (unused) were cleaned with a micro-90 detergent solution to remove any debris from the surface.

III. The laser in the AFM was aligned after which the photo-detector was adjusted using the settings on the screen.

IV. The sample was loaded into the chamber and brought into focus.
   - Initial Scan Size: 1µm, Scan Rate: 1 Hz, Scan Angle: 0 X and Y Offsets: 0

V. The tapping mode was commenced (cantilever tune) and the probe was engaged.

VI. The surface roughness was measured through the use of the tapping mode of AFM on three separate measurements of each surface which are fed back to the computer programme attached.

VII. The images and measurements were saved after each reading.

VIII. The mean surface roughness (nanoscale roughness - Ra) was calculated by determining the roughness of the surface spanning the diameter of the coupon. This assessment was repeated in triplicate.

2.5.4. Validation of Removal Techniques

In order to optimize biofilm removal the use of alternative sonication conditions were investigated.

I. Biofilm was developed using *P. aeruginosa* ATCC 27852 on the five surfaces using the method described in section 2.5.1. After biofilm formation for 48 hours the surfaces were sonicated at 50 kHz in 10ml PBS. The quantity of viable cells was determined using the plate count method as previously described.

II. The sonication conditions of 50 kHz was chosen as the initial sonication setting based on previously published material using the same sonication bath for removal of biofilm cells [186].

III. Biofilm was developed using *S.* Agona S08-0601 on the 5 surfaces using the method described in section 2.5.1. After biofilm formation the surfaces were immersed in PBS and sonicated at 10, 15, 20, 25 KHz.

Swabbing the surfaces was also investigated as an alternative method for biofilm removal as previously described in the literature [71, 150].

I. Biofilm was developed using *S.* Agona S08-0601 on the 5 surfaces using the method described in section 2.5.1.

II. The surface of each coupon was swabbed using a pre-moistened sterile cotton swab after which the content was mixed in 10ml of sterile PBS.

III. The 10ml of PBS was vortexed for 30 seconds at high speed followed by serial dilutions and use of the plate count method as previously described (section 2.5.1).

Figure 2.1: Image of CBR and biofilm substrata (coupons)

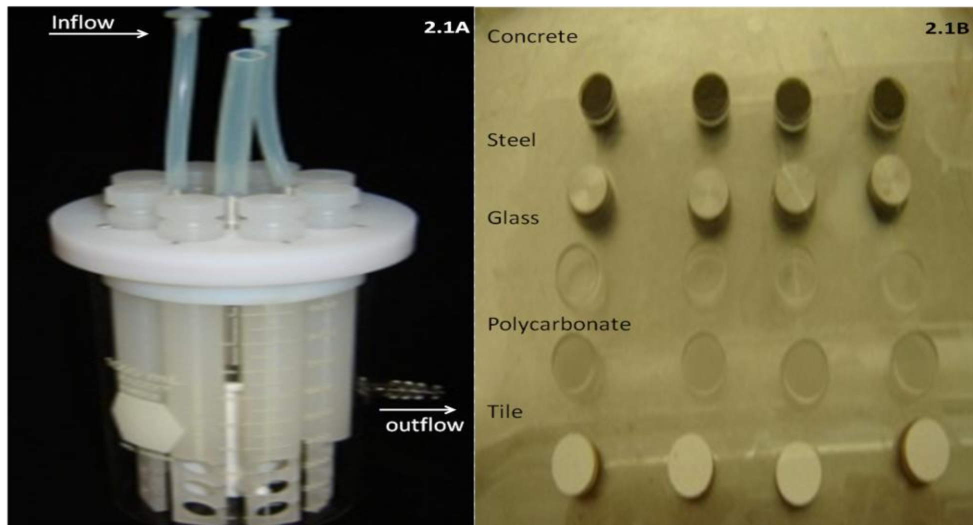


Figure 2.1A shows an image of the CBR with the inflow and outflow ports outlined.

Figure 2.1B shows an image of the concrete, steel, glass, polycarbonate and tile surfaces.

Figure 2.2: Image of the continuous flow system

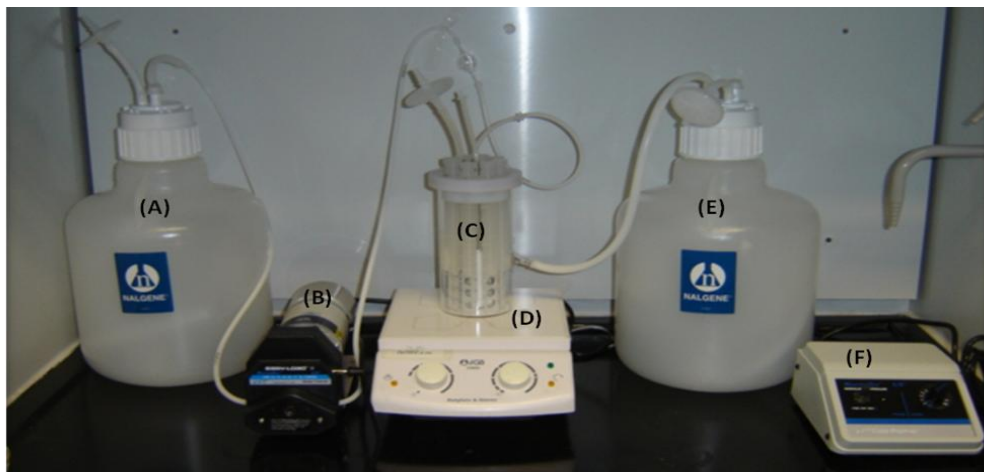


Figure 2.2 illustrates the layout of the CBR system including the carboy used for sterile media **(A),** the pump used to distribute the media into the reactor **(B)**, the CBR **(C)**, the electronic stirrer **(D)**, the outflow carboy used to collect was media **(E)** and the modulator used to control the rate of flow into the CBR **(F)**.

Figure 2.3: A summary of the experimental design

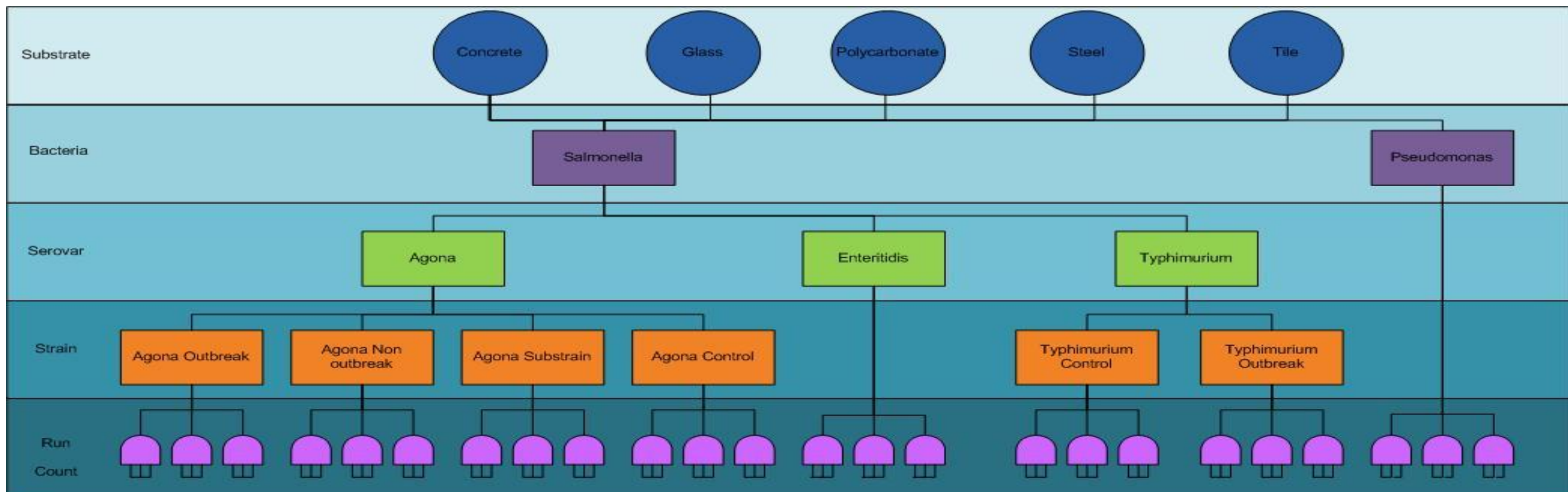


Figure 2.3 illustrates the relationship between the strains examined in this chapter. Essentially a comparative analysis was performed to investigate the differences in biofilm density for *Salmonella enterica* subspecies *enterica* serovars Agona, *S.* Typhimurium and *S.* Enteritidis strains. The difference in biofilm formed depending on source of the strain (outbreak, non outbreak, reference control strains) and the surface to biofilm formed on (concrete, glass, polycarbonate, steel and tile) was also assessed. Design courtesy of Dr. Akke Vellinga.

2.6. Results

2.6.1. Assessment of removal techniques

Table 2.2 displays the density of cells recovered from the five test surfaces after sonication at different output levels (measured in KHz) and two time intervals (30 and 7 minutes). As displayed in Table 2.2 no culturable cells were detected after sonication on high power for 30 minutes. No viable cells were recovered from any of the five surfaces using the high power settings.

Viable cells were recovered from all five surfaces when sonicated at lower power settings of 10, 15, 20 and 25 kHz. The cell counts were lowest at 10 kHz with a slight increase at 15 kHz and highest recovery of cells at 20 kHz. When the sonication conditions were increased to 25 kHz there was a decline in the number of viable cells recovered from the surfaces. As a result, sonication at 20 kHz was performed for the remainder of the experiments described in this thesis. The results displayed in Table 2.2 also indicate that scraping the surface of the coupons with a pre-moistened cotton swab was not an effective method for removing all viable cells.

Table 2.2: Log_{10} density of cells recovered from the surfaces

Strain	Sonication (kHz)	Time (min)	Glass	Steel	Polycarbonate	Tile	Concrete
S08-0601	10	7	2.70	2.87	3.00	3.91	3.99
S08-0601	15	7	2.87	3.24	3.30	4.12	4.17
S08-0601	20	7	6.47	6.57	6.63	7.27	7.34
S08-0601	25	7	5.70	5.87	6.00	7.04	7.22
27853	50	30	ND	ND	ND	ND	ND
S08-0601	Swabbed		2.17	2.30	2.24	2.57	2.47

Table 2.2 illustrates the log_{10} density of cells recovered from the surface after sonication at 10, 15, 20, 25 and 50 kilohertz (kHz). The strains examined include 1 *Salmonella enterica* subspecies *enterica* serovar Agona (S08-0601) and 1 *Pseudomonas aeruginosa* strain (27853). The mean log_{10} density of cells recovered after scraping the surfaces with a sterile swab are also displayed. ND indicates that no cells were detected.

2.6.2. Scanning Electron Microscopy

SEM analysis was also used to assess biofilm removal from the surfaces using the swabbing technique. Figure 2.5 illustrates the biofilm attached to the surface was not successfully removed from the smooth surface (glass) or the porous/rough concrete surface via swabbing.

Figure 2.4: SEM image of glass and concrete demonstrating incomplete biofilm removal

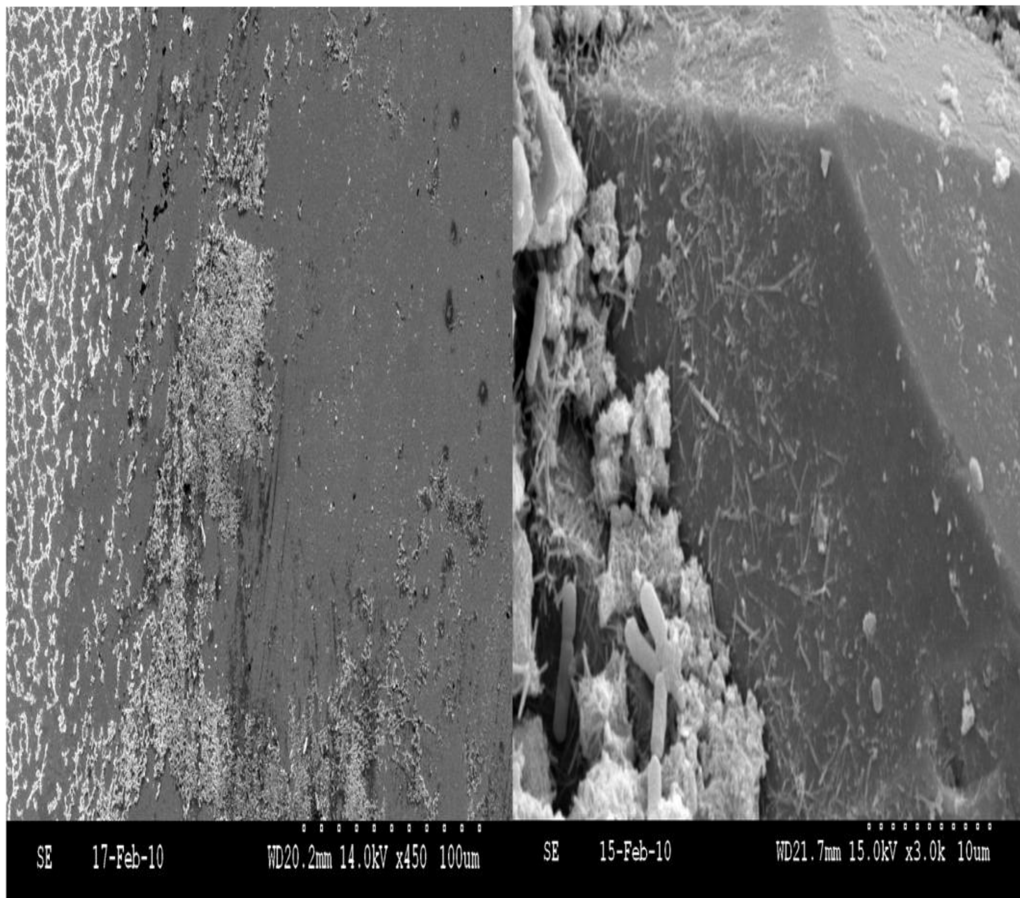


Figure 2.4 demonstrates incomplete removal of the *Salmonella enterica* subspecies enterica serovar Agona S08-0601 from the glass (left) and concrete (right) surfaces after biofilm formation for 48 hours. Removal using a sterile cotton swab was assessed.

2.6.3. SEM Analysis of surfaces to confirm biofilm removal via sonication

All surfaces were examined using SEM before and after sonication for a set of coupons from 1 of the triplicate runs for each strain. SEM was performed on a set of coupons not exposed to sonication to assess biofilm formation on the surface. Post-sonication SEM analysis was performed to assess completeness of removal of cells from the surface. The results indicate that all biofilm cells were removed from the surface after sonication for 12 of the 13 strains examined.

However the *S*. Typhimurium LT2 biofilm was not effectively removed from the surface using the sonication conditions described. Figure 2.5 displays the SEM Images of *S*. Typhimurium LT2 biofilm attached to glass, steel, polycarbonate, tile and concrete after sonication for 7 minutes at 20 kHz. Figure 2.6 displays complete removal of *S*. Typhimurium SL1344 from the surfaces via sonication using the same conditions.

Figure 2.5: Incomplete removal of S. Typhimurium LT 2 on glass, steel, polycarbonate, tile and concrete

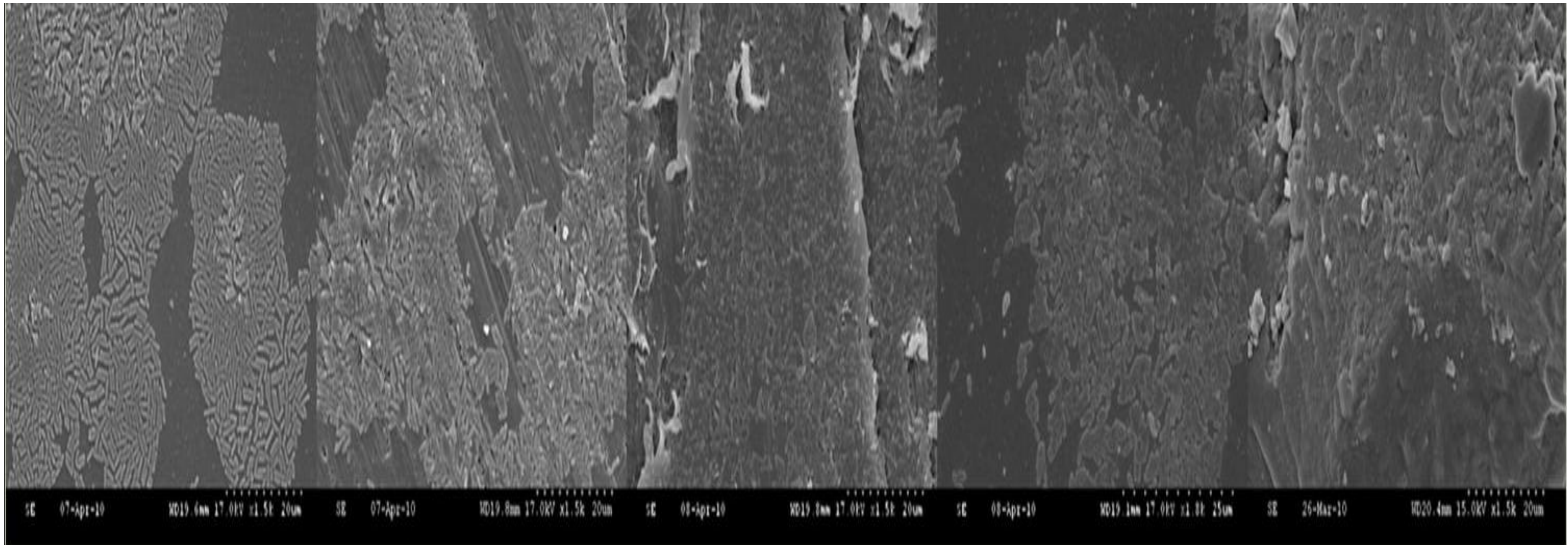


Figure 2.5 displays the SEM Images of *Salmonella enterica* subspecies *enterica* serovar Typhimurium LT2 biofilm attached to glass, steel, polycarbonate, tile and concrete after sonication for 7 minutes at 20 kHz.

Figure 2.6: Complete removal of S. Typhimurium SL1344 from glass, steel polycarbonate, tile and concrete

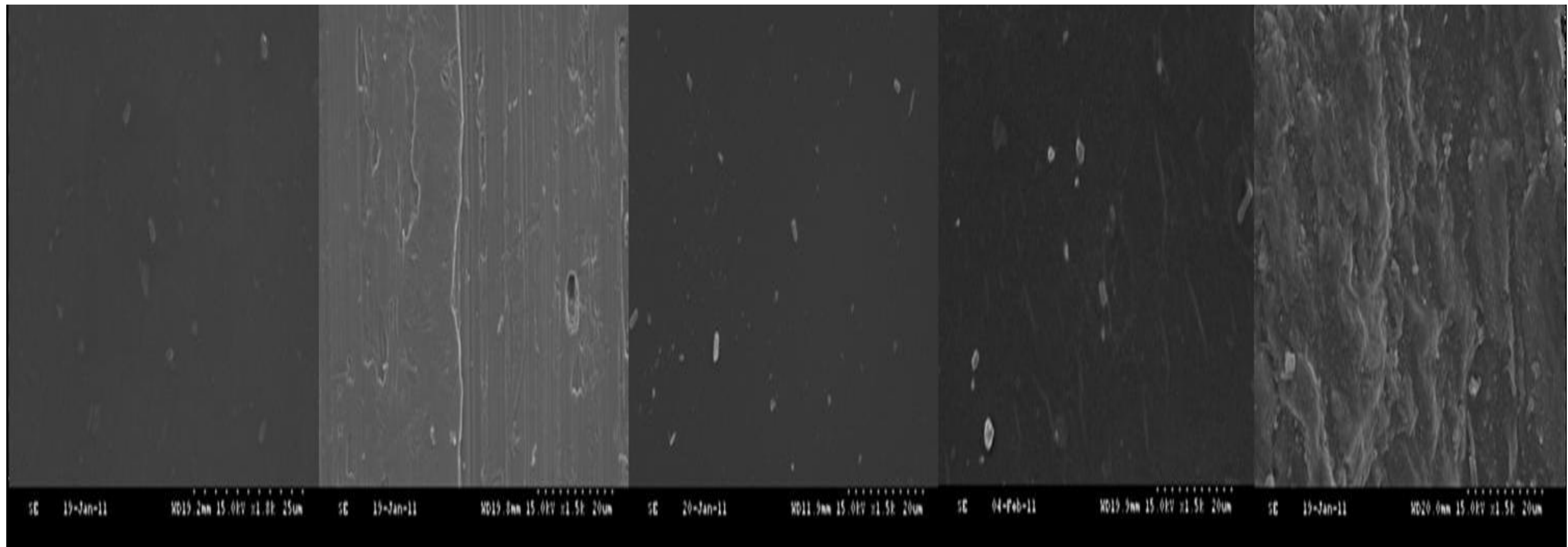


Figure 2.6 displays complete removal of *Salmonella enterica* subspecies *enterica* serovar Typhimurium 1344 biofilm attached to glass, steel, polycarbonate, tile and concrete after sonication for 7 minutes at 20 kHz.

2.6.4. Mean log_{10} density of *S. enterica* biofilm

Each surface type was tested using 3 coupons from each of 3 CBR runs (9 coupons in total) for all strains. As previously discussed *S*. Typhimurium LT2 was not completely removed from the surfaces via sonication. Therefore the mean log_{10} density results for this strain are not incorporated into any comparative work. However the mean log_{10} density of cells removed from the surfaces are listed in Table 2.4 to demonstrate dense biofilm recovered from the surfaces despite incomplete removal.

The layout of the results is based on the aims of the project outlined in chapter one. Firstly to investigate if the *S*. Agona strains SAGOXB.0066 formed a more dense biofilm than other variants of *S.* Agona. To achieve this aim the biofilm density of the *S*. Agona strains linked to the SAGOXB.0066 outbreak were compared to investigate if the strains were similar. Secondly the *S*. Agona strain S08-0601 linked to the outbreak was chosen as a representative strain to compare to the non outbreak related *S*. Agona strains. The mean log_{10} density of *S*. Agona cells recovered from the surfaces is discussed in section 2.6.5.

In order to investigate if *S*. Agona formed a more dense biofilm than other serovar commonly associated with food-borne outbreaks, the *S*. Typhimurium (section 2.6.6) and *S*. Enteritidis (section 2.6.7) strains were compared to each other to investigate any intra-serovar differences between the strains. The mean log_{10} density for each serovar was then examined as described in section 2.6.8.

The density of *S. enterica* biofilm attached to the surfaces was examined in order to investigate if particular surface had a higher propensity to support biofilm growth (section 2.6.9). The relationship between surface roughness and biofilm density was also investigated in section 2.6.10.

2.6.5. Mean $\log_{10}$ density of *S*. Agona biofilm cells

Table 2.3 presents the mean $\log_{10}$ density (CFU/coupon) of *S*. Agona cells attached to glass, stainless steel, polycarbonate, concrete and glazed tile. Table 2.3 also provides an indication of the level of intra-laboratory reproducibility through the use of the standard deviation (SD). The results suggest that *S*. Agona S09-0371 formed a more dense biofilm than all other *S*. Agona strains. The sequenced *S*. Agona reference strain SL483 also formed a dense biofilm on glass, stainless steel and polycarbonate but formed a less dense biofilm than all other strains on concrete and tile. Based on the results presented in Table 2.3 the *S*. Agona strains associated with the outbreak (S08-0601 and S09-0494) do not appear to be more dense biofilm formers than any of the other *S*. Agona strains examined and if anything form a less dense biofilm compared with S09-0371.

Possible differences between the biofilm density for the *S*. Agona strains was further evaluated. Table 2.3 displays the difference in mean $\log_{10}$ density of biofilm produced by the *S*. Agona strains by subtracting the mean $\log_{10}$ density values for the respective strains from each other. The results provided in Table 2.3 indicate that there were only minor differences at best between all strains with PFGE type SAGOXB.0066. As a result the *S*. Agona strain S08-0601 was used as representative of this PFGE type for further comparisons. Based on the results presented in Table 2.3 there appears to be little difference between the *S*. Agona PFGE type SAGOXB.0066 (S08-0601) and the *S*. Agona variant strain (S09-0046 – differs by 2 band based on PFGE analysis) with $p>0.05$ on all 5 surfaces. In relation to comparisons with strains entirely unrelated to the outbreak there was a tendency for the *S*. Agona SAGOXB.0066 related strains to form a less dense biofilm than the other strains tested.

Table 2.3: Mean and standard deviation of all *S.* Agona strains (measured in Mean Log_{10} CFU/coupon)

S. Agona	*N*	Glass			Steel			Polycarbonate			Concrete			Tile		
		Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
***S. Agona (S08-0601)**	**9**	**5.43**	**0.51**		**5.38**	**0.35**		**6.03**	**0.54**		**6.96**	**0.68**		**7.33**	**0.28**	
**S.* Agona (S09-0494)	9	5.41	0.19	0.578	5.80	0.33	0.016	6.33	0.26	0.092	7.08	0.15	0.289	7.56	0.18	0.102
S. Agona (S09-0046)	9	5.25	0.66	0.474	5.53	0.54	0.338	6.07	0.39	0.624	6.99	0.17	0.216	7.26	0.18	0.453
S. Agona (S09-0371)	9	6.16	0.55	0.010	5.59	0.94	0.348	6.81	0.17	0.005	7.47	0.19	0.005	7.59	0.08	0.042
S. Agona (S09-0479)	9	5.63	0.44	0.425	5.56	0.18	0.163	6.43	0.20	0.051	6.88	0.15	0.200	7.10	0.16	0.070
S. Agona (SL483)	9	5.81	0.31	0.068	6.12	0.36	0.002	6.59	0.529	0.100	6.75	0.14	0.145	6.94	0.08	0.006

Table 2.3 displays the mean log_{10} density of cells recovered from the 5 surfaces after 48 hour biofilm development using 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains. The standard deviation of repeated measures (SD) for the nine observations (*N*=9) is also provided. The SAGOXB.0066 outbreak strain S08-0601 which is denoted by bold font in the table, was used to compare the mean log_{10} density of the outbreak strains to the other *S*. Agona strains related to the outbreak (S09-0494) and to the other *S*. Agona strains examined. The difference between the strains is denoted the *P* values in the table. The strains belonging to the SAGOXB.0066 outbreak are indicated by the use of an asterisk *.

2.6.6. Mean log_{10} density of *S.* Typhimurium biofilm cells

Table 2.4 displays the mean log_{10} density values for all *S*. Typhimurium strains. As demonstrated in the results, there was only minor differences in the mean log_{10} density of biofilm recovered from the surfaces for the *S*. Typhimurium strains, despite incomplete removal of the *S*. Typhimurium LT2 (see Figure 2.5 and 2.6).This suggests that the cell counts most likely underestimated the biofilm density. Nevertheless the LT2 strain formed the most dense biofilm on 4 of the 5 surfaces compared with other *S*. Typhimurium strains. However, due to the incomplete removal from the surface the LT2 strain the data for this strain were excluded from all statistical comparisons as the cell counts could not be considered as reflecting the full biofilm density. When the *S*. Typhimurium strain LT2 was excluded from all comparative work a trend towards more dense biofilm formation by the *S*. Typhimurium strain S09-0419 across all 5 surfaces was observed.

Table 2.4 also displays the difference in mean log_{10} densities of *S*. Typhimurium cells recovered from the surfaces (indicated in table as *p* values). The results presented in table 2.4 indicate that there were only marginal difference between the *S*. Typhimurium strains examined and in most is not cases not likely to be significant ($p>0.05$ for 4 of 5 comparisons between SL1344 and S09-0419). The largest difference between strains (difference in mean log_{10} density) was between the two strains S08-0408 and S09-0419, the S09-0419 strain formed a more dense biofilm on 4 of the 5 surfaces ($p<0.05$) but with very little difference on glass (difference of 0.06 CFU/coupon $p=0.964$).

Table 2.4: Mean log$_{10}$ density and standard deviation of *S*. Typhimurium strains (measured in Mean Log$_{10}$ CFU/coupon)

	N	Glass			Steel			Polycarbonate			Concrete			Tile		
		Mean	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***
***S*. Typhimurium (S09-0419)**	**9**	**5.41**	**0.32**		**6.28**	**0.37**		**6.53**	**0.45**		**7.00**	**0.15**		**7.23**	**0.12**	
S. Typhimurium (S08-0408)	9	5.35	0.49	0.964	5.46	0.74	0.012	5.92	0.70	0.034	6.70	0.23	0.034	7.01	0.17	0.012
S. Typhimurium (SL1344)	**9**	5.15	0.62	0.503	5.71	0.35	0.426	6.10	0.33	0.595	6.78	0.18	0.376	7.17	0.12	0.047
S. Typhimurium (LT2)	9	6.37	0.22		6.22	0.26		7.19	0.13		7.04	0.05		7.27	0.17	

Table 2.4 shows the mean log$_{10}$ density of cells recovered from the 5 surfaces after 48 hour biofilm development using 6 *Salmonella enterica* subspecies *enterica* serovar Typhimurium strains. The standard deviation of repeated measures (SD) for the nine observations (*N*=9) is also provided. The *S*. Typhimurium strain S09-0419 which is denoted by bold font in the table, was used to compare the mean log$_{10}$ density the *S*. Typhimurium strains S08-0408 and SL1344. The difference between the strains is denoted the *p* values in the table. The LT2 strain was excluded from all comparisons due to incomplete removal from the surfaces.

Table 2.5: Mean log_{10} density and standard deviation of *S*. Enteritidis strains (measured in Mean Log_{10} CFU/coupon)

	N	Glass			Steel			Polycarbonate			Concrete			Tile		
		Mean	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***
***S*. Enteritidis (S09-0717)**	**9**	**4.85**	**0.55**		**4.73**	**0.50**		**5.20**	**0.495**		**6.43**	**0.34**		**7.02**	**0.16**	
S. Enteritidis (S09-0004)	9	5.61	0.28	0.006	5.71	0.28	0.001	5.72	0.272	0.027	6.74	0.18	0.021	7.02	0.20	0.825

Table 2.5 displays the mean log density of cells recovered from the 5 surfaces after 48 hour biofilm development using 6 *Salmonella enterica* subspecies *enterica* serovar Enteritidis. The standard deviation of repeated measures (SD) for the nine observations (N=9) is also provided. The difference between the strains is denoted the p values in the table.

2.6.7. Mean log_{10} density of *S*. Enteritidis biofilm cells

Table 2.5 compare the mean log_{10} density of *S*. Enteritidis biofilm cells recovered from the surfaces. As illustrated in Table 2.5 the differences between the two strains of *S.* Enteritidis (intra-serovar differences) were generally greater than the intra-serovar differences for the other two serovar groups. The *S*. enteritidis strain S09-0004 formed a more dense biofilm than *S*. Enteritidis S09-0717 on 4 of the 5 surfaces $p<0.05$. However there was no difference between the mean log_{10} density of biofilm cells attached to tile (both mean log_{10} density of 7.02 CFU/coupon, $p=0.825$).

2.6.8. Difference in mean log_{10} density of *S. enterica* serovars

As illustrated in Tables 2.3, 2.4 and 2.5 there were limited differences between most strains within each serovar, particularly *S*. Agona and *S*. Typhimurium (intra-serovar differences). In most cases, any difference in mean log_{10} density was not consistent across all 5 surfaces. Therefore the strains in each serovar were grouped in order to investigate if there were any patterns of inter-serovar differences. Table 2.6 displays the mean log_{10} density of cells recovered from each surface for each of the *S*. *enterica* serovar examined. A *P. aeruginosa* strain ATCC 27853 was included in the investigation to examine *S*. *enterica* biofilm density in comparison to a strain of a different genus. The descriptive results displayed in Table 2.6 indicate that the *P. aeruginosa* strain was a more dense biofilm former on glass, steel and polycarbonate surfaces than any of the *S. enterica* serovars. The *S*. Agona serovar was a more dense biofilm former than *P. Aeruginosa*, the *S*. Typhimurium and *S*. Enteritidis serovars on concrete and tile. The *S*. Enteritidis serovar group formed the least dense biofilm in comparison to all other serovars.

The results displayed in Table 2.7 illustrate the difference between the serovars (mean log_{10} difference). The results indicate there were no consistent differences (on all five surfaces tested) between *S*. Agona and *S*. Typhimurium biofilm density. However the density of *S*. Agona cells recovered from glass, concrete and tile was higher (p<0.05) than *S*. Typhimurium. The density of biofilm cells recovered from all 5 surfaces, was much greater for the *S*. Agona serovar group than the *S*. Enteritidis group (p<0.05 across all 5 surfaces examined). *S*. Typhimurium strains also formed more dense biofilm on all five surfaces than the strains belonging to the *S*. Enteritidis serovar (p<0.05 on 4 of the 5 surfaces – excluding glass).

Table 2.6: Mean log_{10} density of *P. aeruginosa* and *S. enterica* strains (measured in Mean Log_{10} CFU/coupon)

Serovars	*N*	Glass		Steel		Polycarbonate		Concrete		Tile	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
P. aeruginosa	9	5.72	0.57	6.30	0.20	6.61	0.43	6.77	0.43	7.05	0.34
S. Agona	54	5.61	0.54	5.66	0.54	6.38	0.45	7.02	0.38	7.30	0.29
S. Typhimurium	27	5.30	0.49	5.82	0.61	6.18	0.56	6.82	0.22	7.14	0.16
S. Enteritidis	18	5.23	0.58	5.22	0.64	5.46	0.47	6.59	0.31	7.02	0.17

Table 2.6 displays the mean log_{10} density of cells recovered from the 5 surfaces after 48 hour biofilm development with 1 *Pseudomonas aeruginosa* strain, 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 3 *Salmonella enterica* subspecies *enterica* serovar Typhimurium strains and 2 *Salmonella enterica* subspecies *enterica* serovar Enteritidis strains. *N* denotes the number of replicate surfaces examined. The standard deviation of repeated measures (SD) was also calculated.

Table 2.7: Difference between the *S. enterica* serovars (measured in CFU/coupon)

Serovars	*N*	Glass		Steel		Polycarbonate		Concrete		Tile	
		Mean diff.	***P***	**Mean diff.**	***P***	**Mean diff.**	***P***	**Mean diff.**	***P***	**Mean diff.**	***P***
S. Agona – *S. Typhimurium*	54/27	0.31	.025	-0.16	.144	0.20	.176	0.20	.004	0.16	.028
S. Agona— *S*. Enteritidis	54/18	0.38	.031	0.44	.017	0.92	<.000	0.43	<.000	0.28	.001
S. Typhimurium – *S*. Enteritidis	27/18	0.07	.681	0.60	.002	0.72	<.000	0.23	<.008	0.12	.022

Table 2.7 displays the difference between the mean log_{10} density (Mean diff) of cells recovered after biofilm formation the *Salmonella enterica* subspecies *enterica* serovar Agona, *Salmonella enterica* subspecies *enterica* serovar Typhimurium and *Salmonella enterica* subspecies *enterica* serovar Enteritidis strains. The *p* values denotes the significance between the groups.

2.6.9. Difference between the surfaces

The results in Table 2.8 demonstrate that there was large variation in the number of cells recovered from a surface. Table 2.8 demonstrates that tile had a significantly more dense biofilm attached to the surface than glass, steel, polycarbonate and concrete for all serovars tested ($p<0.05$). The smallest difference between surfaces (biofilm recovered from the surface) was between tile and concrete as the mean $\log_{10}$ difference was 0.28, 0.32 and 0.43 CFU/coupon for *S*. Agona, *S*. Typhimurium and *S*. Enteritidis strains, however in all cases the difference was still significantly different ($p<0.05$). The largest difference between the surfaces was between tile and concrete and tile and stainless steel.

Table 2.8: The mean log_{10} density of *S. enterica* serovars on the surfaces (difference between tile and glass, steel, polycarbonate and concrete)

Serovars	*N*	Tile-Glass		Tile-Steel		Tile-Polycarbonate		Tile-Concrete	
		Mean diff	***P***	**Mean diff**	***P***	**Mean diff**	***P***	**Mean diff**	***P***
S. Agona	54/54	1.69	<.000	1.64	<.000	0.92	<.000	0.28	<.000
S. Typhimurium	27/27	1.84	<.000	1.32	<.000	0.96	<.000	0.32	<.000
S. Enteritidis	18/18	1.79	<.000	1.8	<.000	1.56	<.000	0.43	<.000

Table 2.8 displays the difference mean log_{10} density of cells (mean diff) recovered from the surfaces after biofilm development for 48 hours. The mean log_{10} density of cells recovered from tile was compared to the mean log_{10} density of cells recovered from glass, steel, polycarbonate and concrete surfaces after biofilm formation using *Salmonella enterica* subspecies *enterica* serovar Agona, *S*. Typhimurium and *S*. Enteritidis.

2.6.10. Atomic Force Microscopy

The results of Atomic Force Microscopy (AFM) analysis indicate that there was a large difference in the surface roughness of the 5 surfaces. The results indicated that glass was the least rough surface (3.31±0.35) followed by tile (5.33±2.10), steel (20.28±6.80), polycarbonate (110.40±73.89) and concrete (288.60±129.79). As displayed in Figure 2.7 based the mean surface roughness measurements (measured in nanometres) and the mean $\log_{10}$ density of biofilm recovered from the surface (taken from Table 2.6) the surface roughness may contribute to the density of biofilm attached to 4 of the 5 surfaces. However, tile was the 2nd least rough surface but had the most dense biofilm attached to the surface for all strains and repeated experiments.

Figure 2.7: Graph of the surface roughness of each surface and *S. enterica* biofilm density

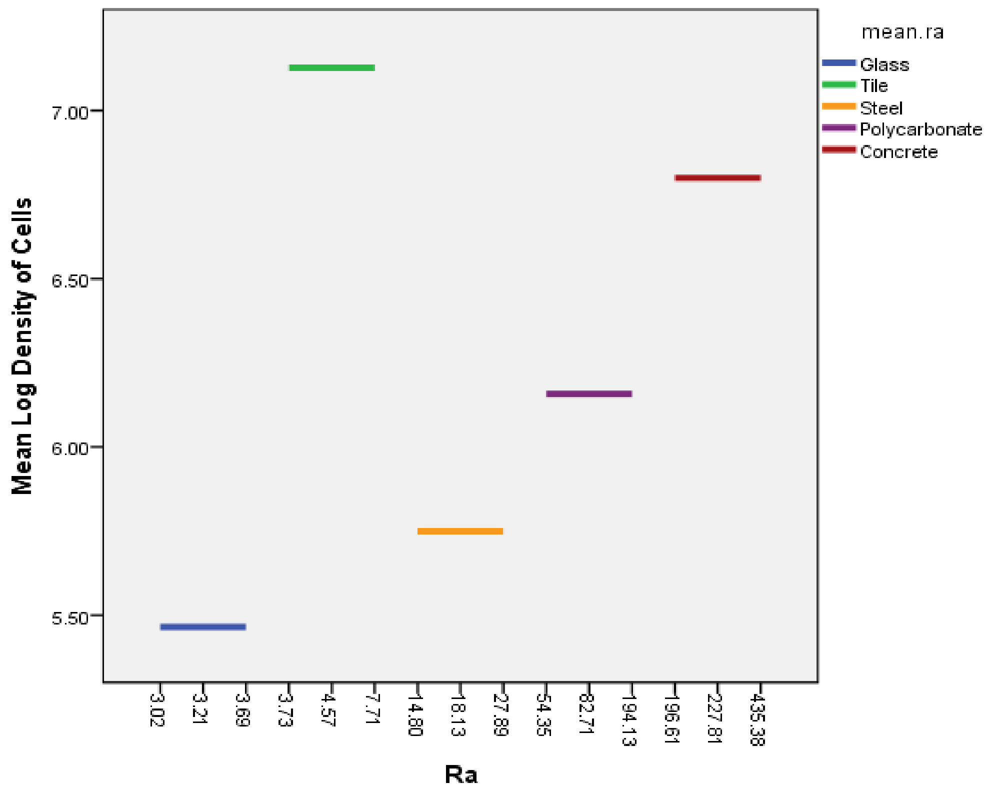


Figure 2.7 illustrates the mean $\log_{10}$ density of *Salmonella enterica* subspecies *enterica* biofilm attached to the 5 surfaces (Y Axis) in comparison to the surface roughness (Ra measured in nanometres) of each surface (X axis). The *S. enterica* strains include the mean $\log_{10}$ density of 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 3 *S.* Typhimurium and 2 *S.* Enteritidis strains listed in Table 2.6. The mean surface roughness (Ra) assessment was repeated in triplicate as indicated from the 3 readings for each surface displayed on the graph.

2.7. Discussion

The results of the quality control experiments suggest that caution is needed when using sonication for biofilm removal. As reported elsewhere researchers should examine the surfaces or biofilm substrata post removal to verify complete removal and confirm validity of the removal method [183]. The results of the quality control experiments indicate that swabbing the surface with a pre-moistened sterile swab was not an effective method for removing all biofilm cells from the entire surface (Table 2.2). Figure 2.4 also illustrates biofilm cells remained on both the smooth (glass) and porous/rough surface (concrete) after swabbing. Moreover cotton deposits remained on the surface after contact with the surface. If this method is chosen in experiments such as those described by Joseph *et al.* [71], it may result in an underestimation of the biofilm density on a test surface. The research published by Joesph *et al.* did not indicate if SEM or any microscopy was performed in order to validate the swabbing method for biofilm removal. Incomplete removal post swabbing may have contributed to the finding of a more dense biofilm recovered from plastic than concrete (cement) [71] which is the opposite to what was found in the research reported in this thesis.

The quality control results also indicated that sonication at 20 kHz was the most appropriate sonication condition for removal of *S. enterica* cells without reducing the number of viable cells. Based on the results presented in this chapter, quality control measures should be performed to ensure cells are not damaged by sonication. Curtin *et al.* used the same model sonication bath previously at 45-49 kHz for 30 minutes to remove *S. epidermidis* biofilm cells from catheter associated surfaces [186]. It is possible that the high sonication conditions may be suitable for gram positive organisms such as *S. epidermidis*. However previous authors have also used higher sonication such as 27 kHz [118] or 42 kHz [72] over short intervals to remove biofilm consisting of gram negative organisms such as *P. aeruginosa*, *Klebsiella pneumoniae* and *Shewanella putrefaciens*. Ceri *et al.*

also used “high” sonication conditions to remove *Escherichia coli* and *P. aeruginosa* ATCC 27853 biofilm attached to a surface, however the exact conditions used were not provided in the article describing the research [114]. However, if high sonication conditions had been chosen without assessment of cells recovery it may have been wrongly assumed that the strains were unable to form a dense biofilm.

The complete removal of viable cells was confirmed with 12 of the 13 strains assessed using scanning electron microscopy. However the biofilm formed by the LT2 strain was not fully removed using the same conditions verified with all other 12 strains. This difference between the LT2 strain and other S. Typhimurium strains is most likely attributable to the described genetic variation of this strain with respect to sigma factor (RpoS) [105]. This genetic difference may contribute to the strain being more dense biofilm former than other strains tested (as seen in tables 2.5) and in the formation of a more adherent biofilm. Due to this deviation of biofilm removal the LT2 strain was excluded from statistical analysis.

The results demonstrate that there is a substantial range of intra-strain variation between the biofilm cell density values achieved through repeated measures (indicated in the respective tables through the use of SD values). Variation between repeated experiments has also been discussed elsewhere [117, 171, 183]. The variation of results is a widely recognised limitation of the biological process with variables such as room temperature, humidity and slight variation in manual procedures (such as biofilm removal and enumeration) contributing to the variation in repeated tests. The same level of variation has also been reported elsewhere using the CBR method [72, 117, 124].

It is not evident from the analysis performed (results displayed Tables 2.3) that *S*. Agona SAGOXB.0066 has a higher propensity to result in a more dense biofilm than any of the other *S*. Agona strains. In fact, in all instances where the difference reaches statistical significance (p<0.05) the outbreak strain had a less dense biofilm on the surface in comparison to the other clinical test strain. Table 2.3 also highlights that there appears to be no significant differences between the outbreak strain and the sub-strain of the outbreak that was also found on the food premises but different by 2 band using PFGE characterisation (S09-0046). The PFGE images of these SAGOXB.0066 is available in Appendix 3, courtesy of the NSSLRL.

When the serovar *S*. Agona was compared to other serovars it was found to be a more dense biofilm former in comparison to *S*. Enteritidis. This observation has also been reported elsewhere. However the differences between *S*. Agona and *S*. Typhimurium were more variable across all 5 surfaces with less indication of a consistent pattern. This suggests that *S*. Agona may be a dense biofilm former compared with *S*. Enteritidis but it may be that the 2 *S*. Enteritidis strains chosen were by chance less dense biofilm formers than the *S*. Enteritidis strains in general. In order to investigate this hypothesis, more strains from the *S.* Enteritidis serovar would need to be included in the analysis. *S*. Enteritidis has been suggested as a dense biofilm former elsewhere [67, 98]. A number of authors have described *Salmonella* biofilm formation with a limited number of strains [71, 102] or with a single serovar [67, 73, 98, 138, 148, 187-190] or using a single biofilm substrata [111, 165, 178, 191]. However in order to validate any findings must confirm their findings on a number of strains or serovars of *S. enterica*.

2.8. Summary

All 13 strains were able to form a biofilm after 48 hours; this may have serious implications for the food industry, who may choose surfaces such as stainless steel as a food contact surface due to ability to clean the surface.

It is evident from the results that tile consistently supported more dense biofilm formation across the 13 strains. This has not been reported elsewhere although tile has been shown to allow attachment and transfer of *S*. Typhimurium to foods [192].

To summarise *S. enterica* biofilm formation may be dependent on serotype, substratum and possibly the origin of the sample may play a role. However the evidence if anything suggests that the outbreak strain *S*. Agona SAGOXB.0066 has a lesser propensity for biofilm formation although given the extent of intra-run variation it is uncertain how much weight can be give to the apparent differences.

The broad range on differences on repeated values of testing the strains on the same surfaces highlights that caution should be used when assigning practical significance to minor differences observed in laboratory models. However it is important to highlight that this variance is similar to what has been reported elsewhere using the CBR, including the manufacturers of the model who devised the optimum conditions for the biofilm development. However, on a number of occasions the work described in previous literature does not incorporate a large range of multiple runs. Perhaps one of the key findings from this work is that due to inter-run variability, the experiments should be replicated on a number of occasions to determine the accuracy of the findings.

Chapter 3

Assessing the density of a 168 hour *S. enterica* biofilm established using the CDC biofilm reactor

3. Abstract

It has been established that *Salmonella enterica* serovar Agona, serovar Typhimurium and serovar Enteritidis strains can form a biofilm on food contact surfaces. However there is limited information on *Salmonella* biofilm development over longer periods of time. A dense biofilm formed over an extended period of time may prove particularly difficult to eradicate in food manufacturing environments. This may result in recurrent outbreaks of particular strains of food-borne pathogens. The purpose of this work is to assess change in density of *Salmonella enterica* biofilm after a longer period of time than what is currently used in many studies. For this element of the thesis the biofilm was allowed to develop over 168 hours (7 days) and culturable counts were performed to investigate change in biofilm density over time. In general, biofilm became more dense after the extended period of time. At 168 hours the *S*. Agona outbreak associated strain (S09-0494) produced a significantly more dense biofilm than the other *S*. Agona SL483 (p=0.004 on all 5 surfaces). The *S*. Agona outbreak strain (S09-0494) also resulted in a greater density of cells recovered from the surface than *S*. Typhimurium S09-0419 (p<0.05 on 4 of 5 surfaces) and SL1344 (p<0.05 on 3 of 5 surfaces). The largest increase in mean $\log_{10}$ density over time was found with the *S*. Enteritidis strains (S09-0717). This is of interest as it may suggest that particular strains which have been considered less dense biofilm formers may be more appropriately considered as slow biofilm formers that form a dense biofilm over an extended period of time.

3.1. Introduction

Salmonella can remain viable on dry surfaces and transfer to other surfaces for over 96 hours after initial inoculation [193]. The presence of organic matter or cracks and crevices may also increase the survival time on surfaces. The ability of *Salmonella* strains to cause persistent contamination over an extended period of time has been reported

previously [163]. These environmental persisters may be related to the formation of a mature biofilm [56]. After a mature biofilm has developed it may be difficult to eradicate the pathogen from the environment due to the number of metabolic processes as outlined in earlier chapters.

3.1.1. Repeated *S. enterica* outbreaks

As discussed in detail in chapter 1 the serovars studied in this research (*S*. Agona, *S*. Enteritidis and *S*. Typhimurium) have been involved in multiple food-borne outbreaks. Repeated isolation of PFGE indistinguishable isolates from a source suggests that the isolates represent a strain that is resident in that niche. Isolation of PFGE indistinguishable isolates from different sources is generally interpreted as indicative of a recent common origin and my help to link cases of infection with a specific niche [194].

Nesse and colleagues analysed *Salmonella* isolated from the environment and the raw ingredients used in four fish feed factories over an extended period of time [163]. All isolates collected over three years were assessed by PFGE and plasmid analysis. In addition, strains taken retrospectively from the factories spanning a ten year period were also examined. It was evident from the results that there were numerous distinguishable PFGE profiles found within the ingredients [163]. Specific clonal lineages of *Salmonella* (based on indistinguishable PFGE profiles) were recognized to persist within the environment over the 3 year period, with the same serovar (no PFGE data) reported in the environment for up to ten years [163].This indicates a particular strain may enter and persist in a premises and that contamination of products may come from this source rather than from raw materials [163].

Following the PFGE analysis of *S. enterica* collected in the fish feed factories the same research group sought to link persistence in the environment with dense biofilm formation [56]. Seven strains were

classified as persistent (4 *S*. Agona, 3 *S*. Montevideo) and 14 presumed non persistent (2 *S*. Agona, 3 *S*. Montevideo, 3 *S*. Senftenberg, 6 *S*. Typhimurium). Biofilm formation was assessed using the microtiter plate method measuring optical density (OD) and biofilm (pellicle formation) at the air-liquid interface. The *S*. Agona and *S*. Montevideo serovars resulted in a more dense biofilm and the biofilm formed more rapidly than the biofilm formed by strains of *S*. Senftenberg and *S.* Typhimurium [56]. Although there were significant limitations in the data in terms of limited assessment of reproducibility of observations which are further discussed in this thesis in chapter 6 where the reproducibility and repeatability of this work is compared the results achieved in this thesis.

3.1.2. Repeated *S*. Agona outbreaks – persistence in the environment

As discussed in chapter 1, the *S*. Agona outbreak strain SAGOXB.0066 was isolated from various food products during the course of the outbreak and traced back to a processing line over the course of the investigation [58]. After a pharmaceutical grade cleaning of the food plant the outbreak strain was no longer identified within the facility. However the SAGOXB.0066 variant (distinguishable by 2 bands) was subsequently isolated from the drains of the campus for an extended period of time. The persistence of the strain prompted this research to assess the relative density of biofilm formed by this strain over an extended period. For purposes of comparison, representatives of the serovars *S*. Typhimurium (including a monophasic variant) and *S*. Enteritidis were also studied.

3.1.3. Biofilm formation over an extended period of time

Most reported work on biofilm is based on studies of biofilm formed over 24 or 48 hours [56, 71, 96, 98, 111, 159, 165, 178, 191]. There is comparatively little work examining biofilm developed over a longer period of time [102, 148-149] and very little or no work comparing biofilm

formation on multiple surfaces by a number of strains over short (≤48 hours) and long periods of time (≥72 hours). In previously published work in this area authors have examined biofilm formation of a single strain over an extended period of time [102, 106, 148-149, 188] or examined the use of two strains [71] or four strains of the same serovar (*S.* Enteritidis) [67] after biofilm formation after a specific duration of time. This is a significant gap in knowledge as it may be that there are important differences between early biofilm and late biofilm. It is likely that late biofilm is more relevant to the situation that can arise in food processing environments when a specific strain is repeatedly isolated over a period of months or years.

3.2. Method – 168 hour biofilm

This section describes investigation of biofilm density when biofilm was established over an extended period of time (168 hours / 7days) and a comparison of density at 48 and 168 hours. Essentially, the method for the 168 hour biofilm was similar to the standardised method (method described in chapter 2) except the period of continuous flow was extended from 24 hours to 144 hours. The strains studied were an *S*. Agona from the outbreak (S09-0494), *S*. Agona reference strain (SL483), *S*. Typhimurium (S09-0419), *S*. Typhimurium reference strains SL1344 and a *S*. Enteritidis (S09-0717). The characteristics of these strains were previously described in Table 2.1 in chapter 2.

3.3. Validation of techniques

Following SEM analysis (see Figure 3.1 and 3.2) it became clear that the entire biofilm was not removed from the surface using the conditions (20 kHz for 7 minutes) previously optimized in chapter 2 (see section 2.3 in chapter 2). In order to improve removal alternative sonication conditions were examined. Following the conditions described in chapter 2, sonication was performed at 20 kHz for 7, 10 and 14 minutes. The suspended biofilm

was plated onto TSA for enumeration as previously described (section 2.5 in chapter 2).

3.4. Results

3.4.1. Biofilm Removal

Based on the SEM images it appears that most of the biofilm was removed from glass followed by tile, while there were more areas of biofilm remaining attached to steel, polycarbonate, and concrete. Table 3.1 displays the number density of cells recovered from the surfaces following sonication for 7, 10 and 14 minutes. The results in Table 3.1 suggest that more cells were recovered from the 5 surfaces after sonication for 7 minutes than after sonication for 10 or 14 minutes. This pattern was evident for both strains examined (SL1344 and S09-0419). Following these results, it was determined that whilst the sonication for 7 minutes did not remove the biofilm completely it did not reduce the number of viable cells. However sonication for longer durations of time impaired cell recovery as displayed in Table 3.1. Therefore sonication at 20 kHz for 7 minutes was maintained as the sonication condition used throughout the work described in this chapter.

Table 3.1: The density of cells recovered from the 5 surfaces after sonication for 7, 10 and 14 minutes

Strain	Sonication (KHz)	Time (min)	Glass	Steel	Polycarbonate	Concrete	Tile
SL1344	20	7	6.24	7.01	6.44	7.28	7.71
SL1344	20	10	6.09	6.87	6.24	7.17	7.59
SL1344	20	14	5.70	6.17	5.40	6.87	7.40
S09-0419	20	7	6.30	6.67	6.44	7.52	7.60
S09-0419	20	10	5.87	6.57	6.17	7.28	7.40
S09-0419	20	14	5.40	6.09	5.70	7.04	7.22

Table 3.1 represent the Log_{10} density of cells recovered from the surfaces (CFU/coupon). Strains examined included *Salmonella enterica* subspecies *enterica* Serovar Typhimurium (SL1344) and *S*. Typhimurium S09-0419. After 168 hour biofilm formation the coupons were sonicated for 20 kiloHertz (kHz) for 1, 10 and 14 minutes (min) in a saline solution followed by enumeration using the plate count method.

Figure 3.1: The mean log_{10} density of cells recovered from the surface after sonication for 7, 10 and 14 minutes

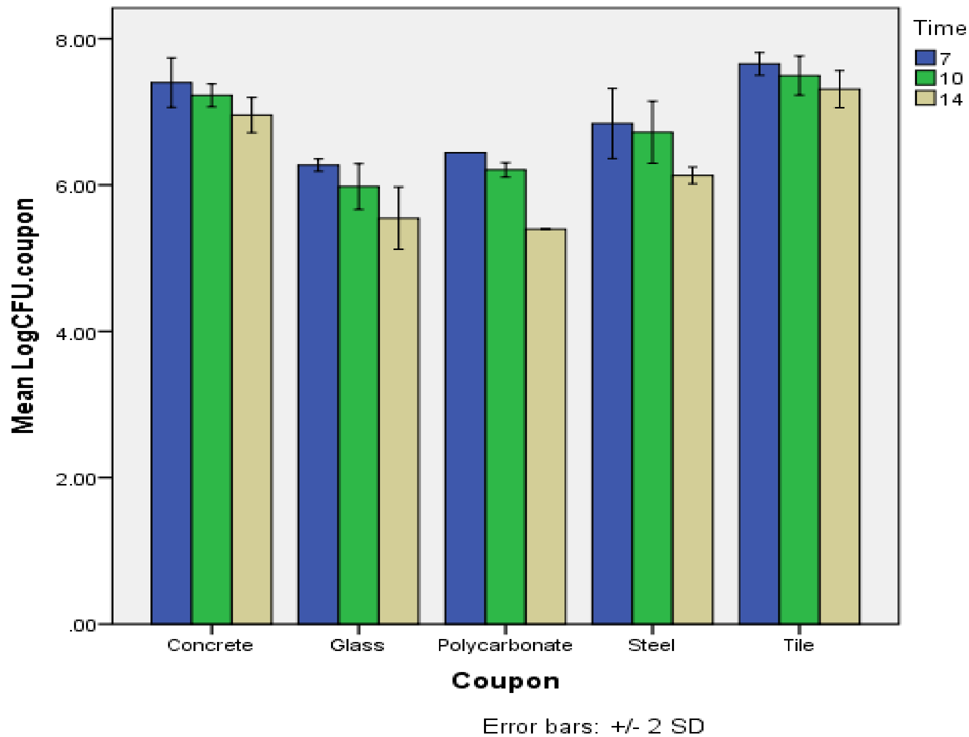


Figure 3.1 displays the mean log_{10} density of cells recovered from the 5 surface after sonication for 7, 10 and 14 minutes. The results displayed are an accumulative mean for both the two *Salmonella enterica* subspecies *enterica* serovar Typhimurium strains (SL1344 and S09-0419). The difference between the strains is denoted by the use of the standard deviation (SD) error bars on the graph. The density is expressed in mean log_{10} density of colony forming units per coupon (CFU/coupon).

Figure 3.2: SEM image demonstrating incomplete removal of *Salmonella* Agona biofilm following sonication

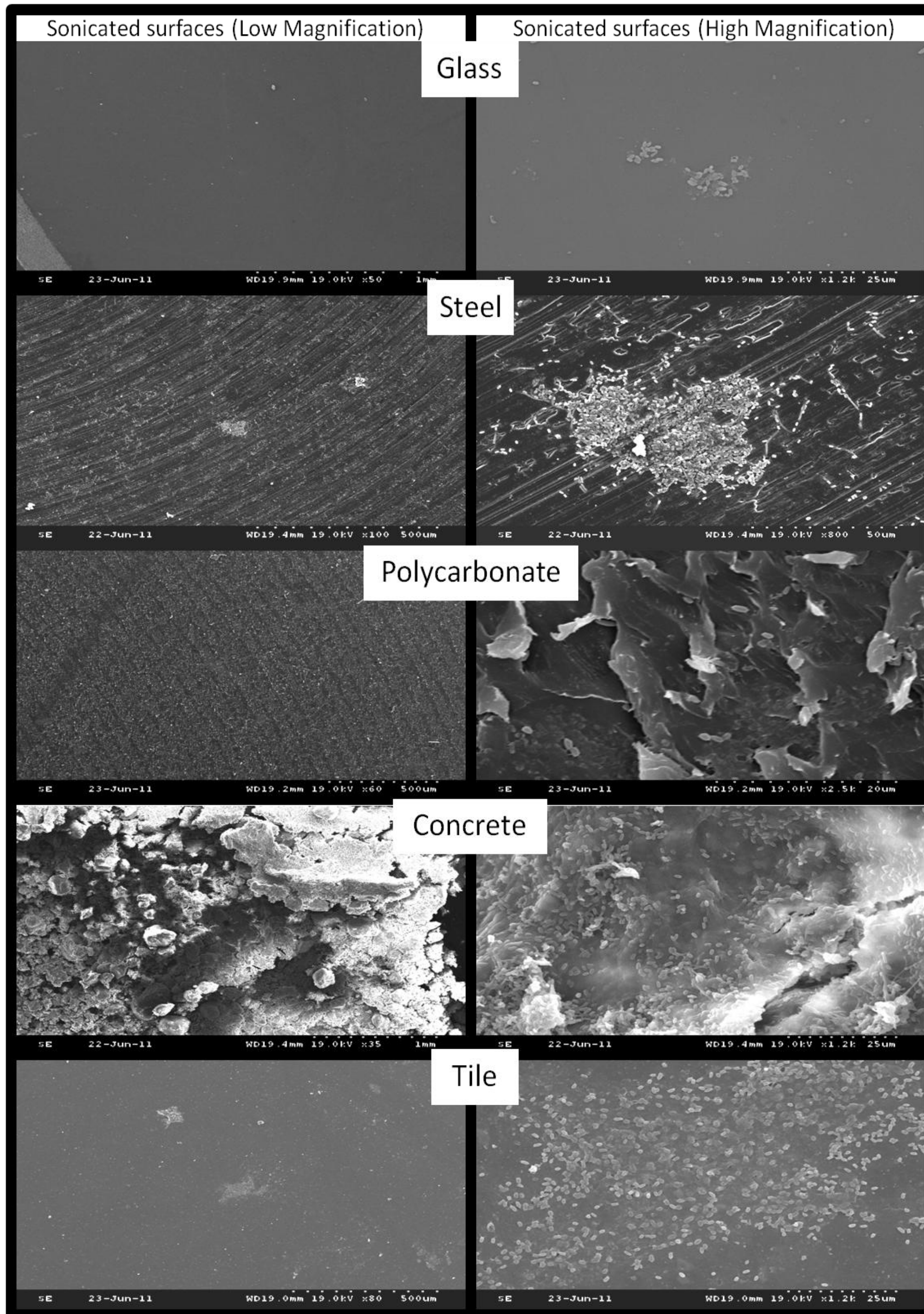


Figure 3.2 displays the scanning electron microscopy (SEM) images demonstrating incomplete removal of *Salmonella* Agona (S09-0494) 168 hour biofilm from the surface following sonication at 20 kiloHertz (kHz) for 7 minutes.

Figure 3.3: SEM image of sonicated and non sonicated steel coupon (complete biofilm) at low magnification

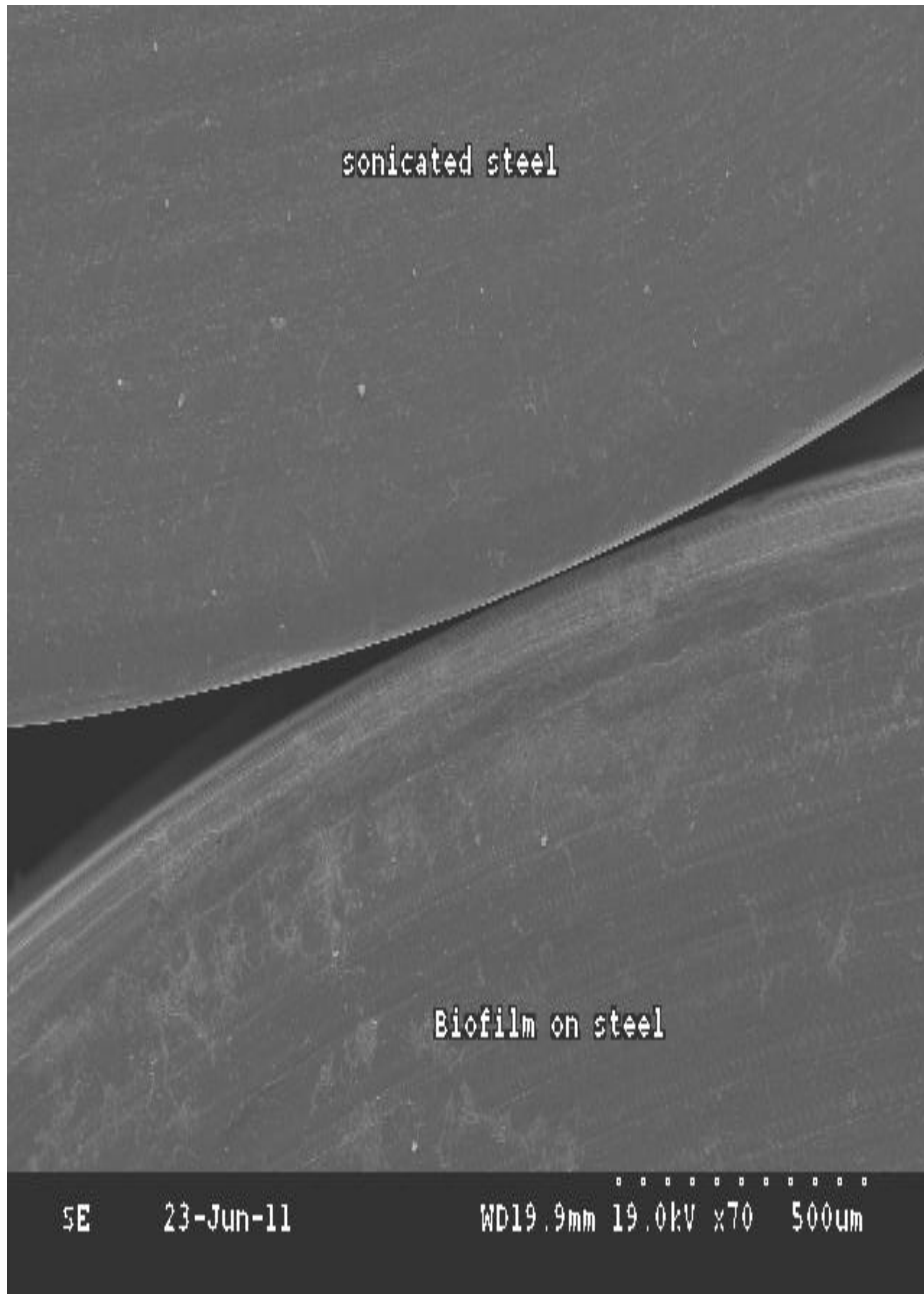


Figure 3.3 displays the biofilm removed from the steel coupon (top image) and the biofilm remaining on the surface of the steel coupon (bottom image). The top coupon was sonicated at 20 kilohertz (kHz) for 7 minutes before being examination in scanning electron microscopy (SEM).

3.4.2. Biofilm density over an extended period of time

Table 3.2 displays the mean log_{10} density of cells recovered from the surfaces after 168 hours biofilm formation. As displayed in Figure 3.2 and 3.3 the 168 hours biofilm was not fully removed from the surface by sonication therefore the viable counts from the 168 hour biofilm most likely underestimate the density of biofilm on the surface. Nevertheless in most instances the mean log_{10} density of cells recovered from the surfaces after 168 hours growth was higher than the mean log_{10} density of cells recovered from the surface when grown for 48 hours. The exceptions were *S*. Agona SL483 on steel and polycarbonate (p<0.05 on the two surfaces) and *S*. Typhimurium S09-0419 on steel, polycarbonate and concrete. The differences in mean log_{10} density of cells recovered from the *S*. Typhimurium S09-0419 biofilm after 48 and 168 hours were minor and not likely of any significance (p>0.05 on all 3 surfaces).

The largest difference between the number of cells recovered from the surfaces after 48 and 168 hour biofilm development was seen for the *S*. Enteritidis strain S09-0717 (p=0.001 for all 5 surfaces). The mean log_{10} density of cells recovered from the surface after *S*. Agona S09-0494 biofilm and S. Typhimurium SL1344 biofilm was established also increased after the extended period of time (p≤0.05 on all 5 surfaces).

The difference in biofilm density after the extended period of time was also apparent through the use of SEM. As displayed in Figure 3.2, the mean log_{10} density of cells attached to all 5 surfaces after 168 hour biofilm formation appears more densely populated than after 48 hour biofilm formation using the S. Agona outbreak strain S09-0494. Moreover the cells of the 168 hour biofilm structure also appear more tightly attached to each other than the biofilm formed after 48 hours which indicates that a mature biofilm may form over the longer period of time.

Table 3.2: Difference between the 48 hour and the 168 hour biofilm (measured in Mean Log_{10} CFU/coupon)

Strain		Glass				Steel				Polycarbonate				Concrete				Tile			
	N	168H	48H	Diff †	*P*	168H	48H	Diff †	*P*	168H	48H	Diff †	*P*	168H	48H	Diff †	*P*	168 H	48H	Diff †	*P*
S09-0494	6/9	7.26	5.41	1.85	**0.001**	6.92	5.80	1.11	**0.001**	6.67	6.33	0.33	**0.011**	7.59	7.08	0.51	**0.001**	7.87	7.56	0.31	**0.018**
SL483	6/9	5.91	5.81	0.10	**0.553**	5.29	6.12	-0.84	**0.007**	5.46	5.59	-0.13	**0.003**	7.08	6.75	0.34	**0.033**	7.52	6.94	0.58	**0.001**
S09-0419	6/9	6.30	5.41	0.89	**0.001**	6.00	6.28	-0.28	**0.087**	6.25	6.53	-0.28	**0.260**	6.93	7.00	-0.06	**0.906**	7.73	7.23	0.49	**0.001**
SL1344	6/9	5.97	5.15	0.83	**0.015**	6.59	5.71	0.87	**0.001**	6.30	6.10	0.20	**0.050**	7.47	6.78	0.69	**0.001**	7.62	7.17	0.44	**0.001**
S09-0717	6/9	6.73	4.85	1.88	**0.001**	6.87	4.73	2.14	**0.001**	6.80	5.20	1.61	**0.001**	7.65	6.43	1.21	**0.001**	8.04	7.02	1.02	**0.001**

Table 3.2 displays the mean log_{10} density of cells recovered from the surfaces after 48 and 168 hour biofilm formation. The strains examined included 2 *Salmonella enterica* subspecies *enterica* serovar Agona strains (S09-0494 and SL483), 2 *S.* Typhimurium strains (S09-0419 and SL1344) and 1 *S.* Enteritidis strain (S09-0717). The difference between the density of cells recovered after the 48 and 168 hours is also displayed through the use of mean difference (Diff= mean density at 168hours –mean density at 48 hours) and *p* values. † Any comparison of the two methods (48 hour and 168 hour biofilm development) needs to be made with a great degree of caution as the 148 hour biofilm was not fully removed from the surface (See SEM images Figure 3.2 and 3.3 for illustration of biofilm remaining on surface). However in most instances the mean log density of cells recovered from the surfaces after 168 hours growth was still higher than the mean log_{10} density of cells recovered from the surface when grown for 48 hours.

Figure 3.4: SEM images *S.* Agona biofilm established at 48 and 168 hours.

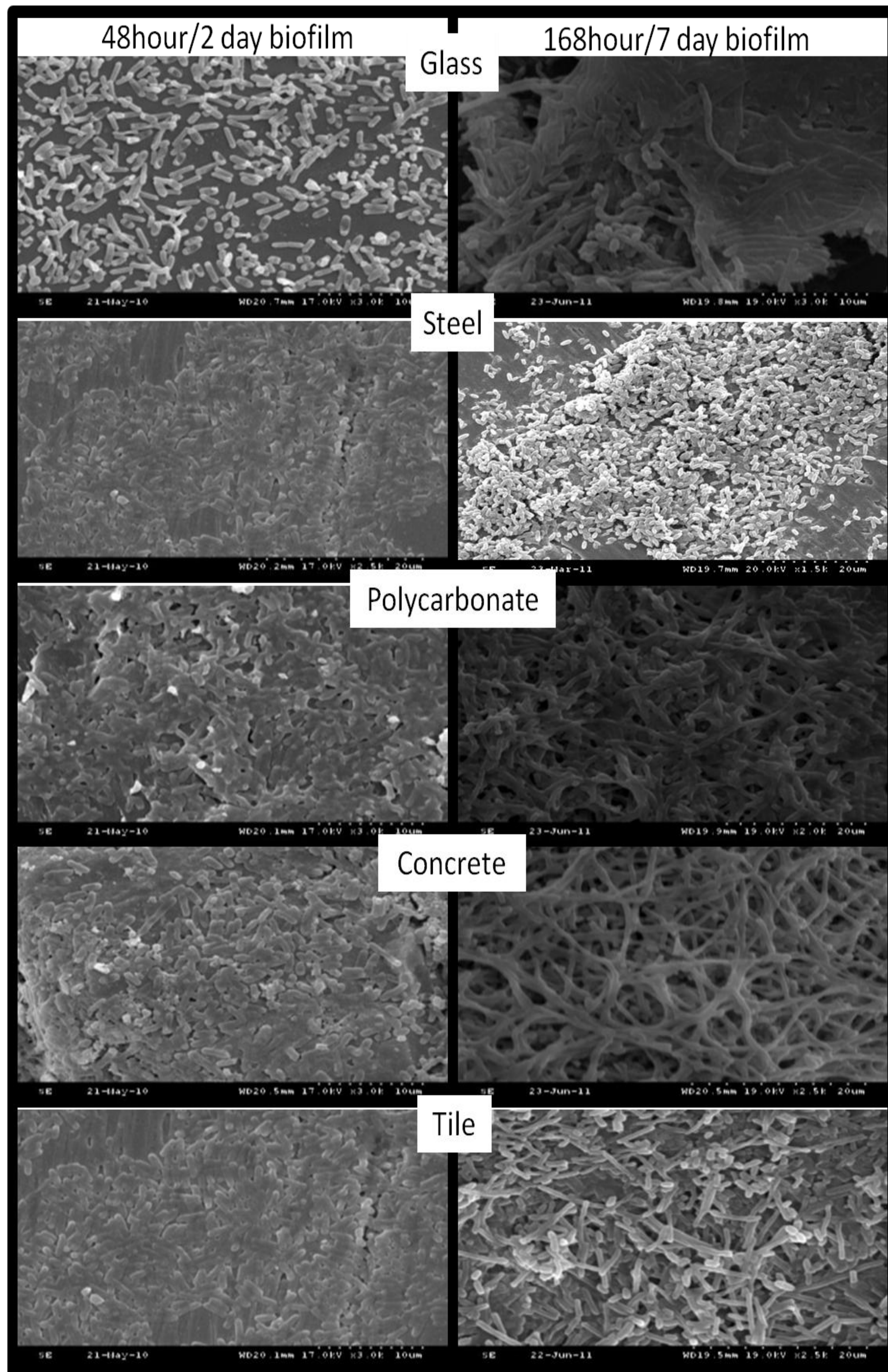


Figure 3.4 displays SEM images *Salmonella* Agona (S09-0494) biofilm established over 48 and 168 hours on glass, steel, polycarbonate, concrete and tile surfaces. Images displayed were taken at high magnification of x2.5-3.0k (k represents x1000 magnification).

3.4.3. Intra-serovar variation in biofilm recovered after 168 hours

Table 3.3 displays the mean $\log_{10}$ density of biofilm recovered from the 5 surfaces after 168 hour biofilm formation. As displayed, more cells were recovered from the *S*. Agona outbreak strain S09-0494 biofilm than the biofilm formed by the *S*. Agona reference strain SL483 on all 5 surfaces (p=0.004 on all 5 surfaces). There was over a 1 $\log_{10}$ CFU/coupon difference in the number of cells recovered from the S. Agona S09-0494 in comparison to the S. Agona SL483 strain when the biofilm was formed on glass, steel, polycarbonate when the biofilm was formed over 168 hours. The differences between *S*. Typhimurium S09-0419 and *S*. Typhimurium reference strain SL1344 were less consistent as more cells were only recovered from the *S*. Typhimurium S09-0419 biofilm on glass and tile.

Table 3.3: Intra-serovar differences in mean $\log_{10}$ density of cells recovered from 168 hour biofilm (measured in Mean Log_{10} CFU/coupon)

		Glass			Steel			Polycarbonate			Concrete			Tile		
		Mean	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***
***S*. Agona (S09-0494)**	**6**	**7.26**	**0.14**		**6.92**	**0.39**		**6.67**	**0.12**		**7.59**	**0.13**		**7.87**	**0.17**	
S. Agona (SL483)	6	5.91	0.31	0.004	5.29	0.53	0.004	5.46	0.55	0.004	7.08	0.28	0.004	7.52	0.08	0.004
***S*. Typhimurium (S09-0419)**	**6**	**6.30**	**0.19**		**6.00**	**0.38**		**6.25**	**0.25**		**6.93**	**0.30**		**7.73**	**0.06**	
S. Typhimurium (SL1344)	6	5.97	0.38	0.107	6.59	0.26	0.004	6.30	0.32	0.518	7.47	0.11	0.004	7.62	0.09	0.016
S. Enteritidis (S09-0717)	6	6.73	0.60		6.87	0.41		6.80	0.31		7.65	0.09		8.04	0.02	

Table 3.3 displays the mean $\log_{10}$ density of cells recovered from the surfaces after 168 hour biofilm formation. The table incorporates the mean $\log_{10}$ density and standard deviation of the mean (SD) of cells recovered from biofilm by 2 *Salmonella enterica* subspecies *enterica* serovar Agona strains (S09-0494 and SL483), 2 *S.* Typhimurium strains (S09-0419 and SL1344). The differences between the strains in each serovar (intra-serovar) were also compared (significance of the differences between the strains denoted by *P* value). The mean $\log_{10}$ density and SD are also included for the *S*. Enteritidis strain S09-0717.

3.4.4. Strain variation in *Salmonella* biofilm formation over 168 hours

The results displayed in Table 3.3 show the inter-strain difference between the mean $\log_{10}$ density of cells recovered from the surfaces after 168 hour biofilm formation. The results indicate that more cells were recovered from biofilm formed by the *S*. Agona outbreak strain S09-0494 than the *S*. Typhimurium strains S09-0419 and SL1344 on all 5 surfaces (p<0.05 on most surface). There were only marginal differences between the density of cells recovered from the *S*. Agona outbreak strain S09-0494 biofilm and *S*. Enteritidis S09-0717 biofilm. As indicated previously in Table 3.2, more cells were recovered from the biofilm formed by *S*. Enteritidis S09-0717 than the biofilm formed by *S.* Typhimurium strains. As a result the difference between the mean $\log_{10}$ density of cells recovered from *S*. Enteritidis S09-0717 and *S*. Typhimurium S09-0419 and *S*. Typhimurium SL1344 were also compared. The results displayed in Table 3.4 also indicate that *S*. Enteritidis formed a more dense biofilm than *S*. Typhimurium S09-0419 and SL1344 (p<0.05 on 4 of 5 surfaces for each strain).

Table 3.4: Inter-strain variation of density of recovered from 168 hours

		Glass			Steel			Polycarbonate			Concrete			Tile		
	N	**Mean**	**Diff**	***P***	**Mean**	**Diff**	***P***	**Mean**	**Diff**	***P***	**Mean**	**Diff**	***P***	**Mean**	**Diff**	***P***
***S.* Agona (S09-0494)**		**7.26**	-	-	**6.92**	-	-	**6.67**	-	-	**7.59**	-	-	**7.87**	-	-
S. Typhimurium (S09-0419)	6	6.30	0.96	0.004	6.00	0.92	0.004	6.25	0.42	0.008	6.93	0.66	0.004	7.73	0.14	0.262
S. Typhimurium (SL1344)	6	5.97	1.29	0.004	6.59	0.33	0.087	6.30	0.37	0.012	7.47	0.12	0.077	7.62	0.25	0.004
S. Enteritidis (S09-0717)	6	6.73	0.53	0.128	6.87	0.05	0.81	6.80	-0.13	0.261	7.65	-0.06	0.262	8.04	-0.17	0.043
***S.* Enteritidis (S09-0717)**	**6**	**6.73**	-	-	**6.87**	-	-	**6.80**	-	-	**7.65**	-	-	**8.04**	-	-
S. Typhimurium (S09-0419)	6	6.30	0.43	0.228	6.00	0.87	0.013	6.25	0.55	0.01	6.93	0.72	0.004	7.73	0.31	0.004
S. Typhimurium (SL1344)	6	5.97	0.76	0.045	6.59	0.28	0.107	6.30	0.5	0.029	7.47	0.18	0.016	7.62	0.42	0.004

Table 3.4 displays the results of inter-strain comparisons *Salmonella enterica.* The mean $\log_{10}$ density of cells recovered from *Salmonella enterica* subspecies *enterica* serovar Agona outbreak strains S09-0494 (highlighted in bold font) was compared to 2 *S.* Typhimurium strains (S09-0419 and SL1344) and *S.* Enteritidis S09-0717 though the assessing difference in mean $\log_{10}$ density values (Diff) and the significance attributed (denoted by *p*). The *S*. Enteritidis strain S09-0717 was also compared to the 2 *S*. Typhimurium strains S09-419 and SL1344.

3.4.5. The density of biofilm on contact surfaces

The results displayed in Table 3.5 indicate that similar to what was observed in chapter 2 that tile supported more dense biofilm than glass, steel, polycarbonate and concrete (*p*<0.05 in all comparisons). The mean log_{10} density of cells recovered from tile was greater than the mean log_{10} density of cells recovered from the all other 4 surfaces for all 5 *S. enterica* strains examined including all replicated experiments.

The results displayed in Table 3.5 shows the difference in mean log_{10} density of cells recovered from tile subtracted from the mean log_{10} density of glass, steel, polycarbonate and concrete.

Table 3.5: The difference in mean log_{10} density of cells recovered from tile and the 4 other surfaces (measured in Mean Log_{10} CFU/coupon)

		Tile							
		Glass		Steel		Polycarbonate		Concrete	
Strain	N	Diff	*P*	Diff	*P*	Diff	*P*	Diff	*P*
S. Agona S09-0494	6/6	0.61	0.004	0.95	0.004	1.2	0.004	0.28	0.016
S. Agona SL483	6/6	1.61	0.004	2.23	0.004	2.06	0.004	0.44	0.004
S. Typhimurium S09-0419	6/6	1.61	0.004	2.23	0.004	2.06	0.004	0.44	0.004
S. Typhimurium SL1344	6/6	1.65	0.004	1.03	0.004	1.32	0.004	0.15	0.024
S. Enteritidis S09-0717	6/6	1.31	0.004	1.17	0.004	1.24	0.004	0.39	0.004

Table 3.5 displays the difference in mean log_{10} density of cells recovered tile and from the 4 other surfaces after biofilm formation for 168 hours. The difference (Diff) was calculated by subtracting the mean log_{10} density of cells recovered from tile from the mean log_{10} density of cells recovered from each of the other surface for each of the 5 strains examined. The strains incorporated 2 *Salmonella enterica* subspecies *enterica* serovar Agona strains (S09-0494 and SL483), 2 *S*. Typhimurium strains (S09-0419 and SL1344) and 1 *S*. Enteritidis S09-0717. The difference (Diff) was calculated by subtraction the mean log_{10} density of cells recovered from glass, steel polycarbonate and concrete from the mean log_{10} density of cells recovered from tile. The significance of the differences is also denoted by the *p* values in table 3.7.

3.5. Discussion

As illustrated in the SEM images provided in the results (Figure 3.2) the biofilm was not completely removed from the five surfaces examined (glass, steel, polycarbonate, tile and concrete). This suggests that the biofilm formed after 168 hours may be more tightly attached to the surface than the 48 hour biofilm. As a result the biofilm was more difficult to detach and recover using the conditions validated previously for the 48 hour biofilm. The finding of *Salmonella* biofilm remaining on the surface after sonication limits the validity of assessing 168 hour biofilm density by enumeration of recovered cells. However, increasing the duration of sonication or the intensity of sonication used (kHz) resulted in a reduction in the number of cells recovered from the surface. Also, as discussed in chapter 2, alternative options such as confocal microscopy and grid counting were not possible on all surfaces. Therefore viable plate counting post sonication remained the most appropriate method for determining the number of cells remaining on the surface.

A limitation of some published work is that a number of authors have not provided details on validation of biofilm removal techniques via sonication [72, 114, 118, 122, 124] including authors removing *S. enterica* biofilm formation by other techniques [71, 105, 150]. The use of SEM analysis and quality control experiments to validate conditions chosen allows researchers to ensure all limitations relating to the data are apparent. Hamilton *et al*. also highlighted the importance of validating biofilm removal and disaggregation methods. Incorrect reporting of biofilm density can have major implications, such as an under-estimation of biofilm cells attached to a surface [183].

Giaouris *et al.* also confirmed that a 7 day *S.* Enteritidis biofilm was not removed from the stainless steel surface via bead vortexing [188]. The

authors suggested that plate counting in addition to conductance measurements (assessment of kinetics in broth used for growth) allowed a more accurate estimate of number of biofilm cells [188]. However the conductance measurement may not be possible if other chemicals such as disinfectant agents are added. It may also be possible for the kinetics to be disrupted while the cells remain viable but not culturable [161].

Previous authors have suggested that sonication results in the recovery of more biofilm cells attached to a surface than scraping [195]. With the exclusion of *S. epidermidis* on steel, Bjerkan *et al.* reported that sonication recovered more *S. aureus, Enterococcus faecalis,* and *Propionibacterium acnes* biofilm cells from steel and titanium implants than scraping methods [195]. The finding that sonication resulted in recovery of more cells than scraping or swabbing the surface was also validated through the use of SEM as discussed in chapter 2. Kobayashi *et al.* demonstrated that sonication with vortexing was more effective for recovering *S. aureus* biofilm attached to prosthetic device than vortexing alone [196]. Moreover, through the plate count and molecular biology techniques Kobayasi *et al.* reported that the number of biofilm cells recovered from the surface correlated with an increase in the sonication time allowed, under which sonication for 30 minutes at 40 kHz resulted in the highest yield of cells [196].

Monsen evaluated the effect of sonication on viable cells of a number of gram negative and gram positive organisms [181]. Similar to what was reported in this thesis, Monsen *et al.* indicated that for most of the bacteria sonication for 7 minutes at 20 kHz resulted in no reduction in the number of viable cells recovered [181]. This suggests that the longer time periods may have resulted in reducing the number of viable cells possibly trough the disruptive nature of the sonication process.

As discussed in section 3.4.2 the density of cells recovered from the surfaces increased after 168 hour biofilm development. This trend was apparent for most strains examined. However the density of *S*. Enteritidis S09-0717 cells recovered from the 5 surface after 168 hours was much greater than the density of S09-0717 cells recovered from the 5 surface after 48 hours ($p<0.001$). This is of interest as based on the 48 hour biofilm two *S*. Enteritidis strains (S09-0717 and S09-0004) were considered as forming less dense biofilm than other serovars. However based on the results presented in chapter 3 it may be more appropriate to consider *S*. Enteritidis as a slow biofilm former because over an extended period of time the *S*. Enteritidis strain formed a more dense biofilm than the 2 *S*. Typhimurium strains and the *S*. Agona reference strain. However, as only 1 *S*. Enteritidis strain was included in this assessment caution should be exercised as it is not evident that this observation would be true if more strains of *S*. Enteritidis were used.

Previous authors have also suggested that *S*. Enteritidis can form a dense biofilm over an extended period of time, however most of this research has only examined 1 strain of *S*. Enteritidis [73, 102, 106, 148, 188]. Stocki *et al.* also examined biofilm formation of 2 *S*. Enteritidis strains on plastic conveyor belts frequently associated with poultry/egg processing farms [67]. Rodrigues *et al*. examined biofilm formation of 4 strains of *S*. Enteritidis on kitchen surfaces including granite, marble and types of silestone [98]. However, these findings were also only limited to the *S*. Enteritidis serovar. It may have been of use to examine biofilm formation of multiple strains and serovars of *Salmonella* on food contact surfaces as the density of biofilm formed may both be strain specific or surface dependent which may hold significant relevance to the food processing industry.

S. Enteritidis was a less dense biofilm former than strains of *S*. Agona and *S*. Enteritidis after 48 hours. Interestingly, had the *S*. Enteritidis strain been excluded from the work examining biofilm formation over the extended period of time it may have been assumed that only the *S*. Agona strain formed a significantly more dense biofilm on all 5 surfaces than the other strains of *S*. Agona and *S*. Typhimurium. This finding emphasises the importance of including multiple strains and serovars in biofilm studies.

Furthermore, there may also be use in investigating the density of biofilm formed by multiple strains within each serovar. As clearly outlined in section 3.4.3 the *S*. Agona strain related to the outbreak S09-0494 formed a more dense biofilm than the *S*. Agona reference strain SL483 (p=0.004). This finding suggests that the sole use of only reference strains may be of limited use in biofilm related studies considering outbreak associated strains may form a significantly more dense biofilm. Previous authors have examined biofilm formation of multiple strains and serovars over an extended period of time using a microtitre plate based system [56, 197]. However, there are also limitations to the use of this method such as limited surface area. The use of the microtitre plate based method is discussed in further detail in chapter 5.

Overall, the results presented in this chapter indicate that in most cases *Salmonella* biofilm density increases after an extended period of time. Korber and colleagues also found that *S*. Enteritidis biofilm increased marginally in thickness over an extended period of time (72 hours) using a flow cell device [102]. Similarly, Chorianopoulos also reported that the density of S. Enteritidis biofilm formation increased over 72 hours [148]. Mangalappalli-Illathu *et al.* also demonstrated *S*. Enteritis biofilm density increased over time (7 days) using a flow cell device [189]. These findings are broadly consistent with results presented here, although the extent of

increased biofilm appeared more significant in the results presented in this chapter ($p<0.05$). However, as previous discussed it may have been of use to investigate if these findings were true for more than 1 strain of *S*. Enteritidis.

In contrast to the results presented in this chapter Wong *et al.* indicated that *S.* Typhimurium biofilm density did not increase over an extended period of time (7 days) using the MBEC method [149]. However, this method (similar to microtitre plate method) may not support extended biofilm growth possibly due to a smaller surface area available. Moreover, the authors sonicated the coupons of high settings for 5 minutes post treatment with the disinfectants. As indicated in the work presented in chapter 2, sonication at high settings may reduce the number of viable cells associated with a biofilm. Moreover, the surfaces were not inspected to confirm complete removal, although a previous publication by the group examined the arrangement a 3 day biofilm on the peg surfaces without treatment or sonication removal [198]. In light of this, it is possible that some of the biofilm cells that formed after 3 days may not have been removed from the surfaces, which may elucidate why the authors found no significant difference in the density over the longer period of time [149].

The research presented in this chapter found that in most instances a *Salmonella* biofilm increased in density over the extended period of time of 168 hours. Previous authors have suggested that biofilm growth is optimized after 48 hours using the CDC biofilm reactor [72]. This could have serious implications if biofilm formation is only investigated for a shorter period of time as it may not reflect real life conditions where a biofilm may continue to grow if undetected for an extended period of time.

3.6. Summary

The research presented in this chapter demonstrates that the density of cells recovered from an *S. enterica* biofilm increased over an extended period of time of 168 hours in comparison to the standardised method of 48 hours recommended for using with the CBR and other methods. The increase in cell density was also confirmed through the use of SEM. Secondly, it was evident from the use of SEM and altered sonication conditions that the biofilm was also more tightly attached to the surface after biofilm development over the extended period of time.

Previous authors have suggested that biofilm growth is optimized after 48 hours. This could have serious consequences relating to research involving food-borne pathogens as the research may not reflect real life conditions where a biofilm may continue to grow over time if undetected.

Previous research in this area has tended to examine a limited number of strains or a number of strains from the 1 serovar. The research provided in this chapter suggests that it may be important to incorporate a number of strains and serovars into studies investigating *Salmonella* biofilm formation. Similar to chapter 2, the work presented in this chapter also found that more cells were recovered from tile than other surfaces such as plastic which is commonly used in biofilm studies.

However, a key limitation remains evident in the research described in this chapter as 168 hour biofilm was not fully recovered from the coupons. As a result, incomplete removal decreases the validity of using a plate count method to determine the density of biofilm attached to the surface. Incomplete removal also undermines the use of direct comparisons of the density of recovered cells particularly between the biofilm recovered after 48 and 168 hours. The SEM images also provide evidence to suggest differences in the degree of removal of cells from the 5 surfaces after 168 hour biofilm formation.

Chapter 4

Examining the efficacy of disinfectant products against an established *S. enterica* biofilm

4. Abstract

In the previous chapter evidence was presented that the density of *Salmonella enterica* biofilm can continue to increase over an extended period of time after it becomes established. There is evidence to suggest that biofilm formation also enhances bacterial resistance to disinfectant treatment. The survival of cells may result in bacterial re-growth on surfaces in the presence of nutrients and favourable conditions resulting in cross contamination of work-surfaces, equipment or food. This research evaluated the efficacy of sodium hypochlorite, sodium hydroxide and benzalkonium chloride against a standard (48 hours) and extended (168 hours) *S. enterica* biofilm. Sodium hydroxide was effective at eradicating a standard 48 hour *Salmonella* biofilm as no viable cells were detected after a 10 minute contact time. In addition to failure to detect growth by enumeration of cells following biofilm detachment after sodium hydroxide exposure, no growth was detected when the entire biofilm attached to a coupon was placed in broth for 24 hours. However, the same concentration of sodium hydroxide was not effective at eradicating all viable cells from an extended biofilm (168 hours). Sodium hypochlorite and benzalkonium chloride resulted in a reduction of viable cells directly after exposure however neither of the disinfectants were effective at eliminating all viable cells from the 48 hour or 168 hour biofilm. Studies on the efficacy of chemical disinfectants intended for application in the control of biofilm should include assessment against an established biofilm. In particular studies should examine a biofilm formed over an extended period of time as it may be that eradication of established biofilm with standard chemical disinfectants alone is not possible.

4.1. Introduction

Salmonella readily forms biofilm on food contact surfaces in both industrial and domestic settings [130]. *Salmonella* biofilm growth has been examined

through laboratory based models on a diverse range of surfaces including concrete, tiles, stainless steel, glass, silestone, granite, rubber and a multitude of synthetic plastics [97-98]. Control of *Salmonella* biofilm presents a challenge for the food industry. As discussed in chapter 3, *Salmonella* species have been described as environmental persisters [89]. Long-term persistence of *Salmonella* on surfaces is likely to be associated with the ability to form biofilm and withstand disinfectants [56, 134].

Microbial biofilm formation may increase resistance to disinfectants due to failure of disinfectant to penetrate through the proteinaceous extrapolymeric substances formed on the outer layer of the biofilm resulting in less diffusion and interaction with viable cells. Cellular changes such as a reduced growth rate, changes in metabolic activity and up regulation of genes responsible for biofilm formation, lipid formation, cellular transport, chemotaxis and resistance genes may also contribute to resistance to disinfectants and other antimicrobial compounds [102, 105].

Over an extended period of exposure or through repeated intermittent exposure, *Salmonella* can develop acquired resistance to disinfectants [132, 197]. Disinfectants and other antimicrobial products are commonly used for cleaning surfaces and equipment in the food industry to reduce the risk of product contamination. As a result microorganisms which are introduced to premises on raw meat and other products may come into contact with surface disinfectants from an early stage of processing. Furthermore, raw carcasses are frequently sprayed with antimicrobial agents using high pressure water jets or dipping in large water tanks in order to reduce the microbial load on raw products [199]. After the carcasses are sprayed, the antimicrobial products are in direct contact with microorganism on the meat and can also be introduced to the environment in large volumes [102]. If bacteria survive disinfectant treatment, the

outflow of excess disinfectant diluted water may create a reservoir of antimicrobial resistant bacteria circulating in the environment of a food plant. Antimicrobial compounds associated with meat processing and washing include hydrogen peroxide, ozone, chlorine, sodium chlorite, peroxyacetic acid or trisodium phosphate [200].

Post slaughter washing may produce a build-up of antimicrobial compounds meat products or create a reservoir of excess antimicrobial resistant bacteria in the environment of a food processing area. During the surveillance of four slaughter houses, Arguello *et al.* detected *Salmonella* in the majority of areas in all four slaughter houses tested. In 3 of the 4 slaughter houses, the bung droppers (used for cutting the carcasses) were still contaminated with *Salmonella* after application of the processes intended to achieve sterilization [201]. Arguello *et al.* also isolated *Salmonella* from contaminated scalding tanks used to wash the carcasses [201]. The results from the surveillance indicated that sterilization and disinfection procedures in use at the time of testing were not effective [201].

The presence of *Salmonella* after the cleaning and decontamination processes may also indicate a build up of disinfectant resistant bacteria or biofilm formation. Anderson *et al.* investigated the formation of a multi *Pseudomonas* species biofilm inside a polyvinyl chloride pipe (PVC pipe) over the course of eight weeks[202]. The PVC pipe was then treated with disinfectant agents for 7 days at the recommended working concentrations. Sterile water was then used to examine if remaining cells could contaminate the flow-through effluent. As a result of the biofilm formation, the pathogen was found to contaminate the disinfectant agents recovered from the surface and the water used as wash-out effluent for up to 7 days post treatment [202]. The disinfectants used included chlorine, phenolic

acid and quaternary ammonium solutions. Anderson *et al.* suggested that the presence of a glycocalyx (EPS of biofilm) provided protection to cells within the biofilm, as a result the PVC pipe acted as a reservoir to initiate recurring contamination [202].

4.1.1. Methods for testing disinfectant efficacy and bactericidal activity

Disinfectants are often marketed as active against *Salmonella* however these results are often based on suspension test results only [134]. Tests of disinfectant activity against bacteria in suspension may have some relevance for studying the mechanisms by which disinfectants enter and kill cells. However suspension tests are of limited relevance to control of biofilm as they do not mimic real-life conditions in contaminated environments when cells are more frequently attached to surfaces and/or present in biofilm that in suspension [140]. Disinfectants are generally less active against biofilms than against bacteria that are suspended planktonically. However there is both limited and contradictory information on the extent to which disinfectants are effective against biofilms at different stages in maturation [149].

Methods for assessing the efficacy of disinfectants must be reasonably practical to perform, relevant to real life conditions, valid in reaching log_{10} reduction (i.e. ensure no other components are contributing to any observed reduction) and sufficient to withstand any minor environmental changes [183]. Methods for evaluating efficacy of surface disinfectants must also be responsive to changes such as concentration of test agents with accurate enumeration of viable cells of a remaining pathogen and must be reproducible [117]. In order to mimic real life conditions, the disinfectant work described in this chapter is performed using the CDC biofilm reactor model with concrete coupons used as a biofilm substratum. Concrete was chosen as an appropriate substratum as it is commonly

found in the food processing environment and may act as a source of recontamination in food processing plants [203]. Concrete has a high propensity to support biofilm formation using the CDC biofilm reactor model as described previously in this thesis. Biofilm formation on concrete has also been demonstrated elsewhere [71, 141].

4.1.2. The CEN recommendations for reporting disinfectant efficacy

The European Committee for Standardisation (CEN-Comité Européen de Normalisation) have developed protocols and guidelines for assessing the efficacy of disinfectant products sold within the European Union [130]. According to the European suspension test (EN1040) a ≥5 log_{10} kill (99.999%) is necessary to prove the efficacy of a disinfectant against *P. aeruginosa* or *S. aureus* in a planktonic broth suspension [131]. It is recommended that testing is performed at 20°C or room temperature with a contact time of 5 minutes and incubation at 36-37°C. However as the standard EN1040 is based on a suspension tests only it does not require evaluation of the performance of the disinfectant for removal or destruction when the bacterial cells attached to a surface.

The current standard EN13697:2001 requires a ≥4 log_{10} reduction in cells attached to a surface after contact with an antibacterial agent [133]. The procedure should be applicable for cultures of *P. aeruginosa, S. aureus, E. coli* and *Enterococcus hirae.* The test should be performed at room temperature (18-35°C) with a contact time of 5 minutes with optional additional times of 1, 15, 30 and 60 minutes. A suspension equivalent to a 0.5 McFarland (~1.5-5x10^8 CFU/ml) is made from an overnight culture of the test bacteria. A small volume (500µl) of the suspension is added to the face of the surface (stainless steel-grade B finish) within the confines of a petri dish and allowed to dry at 37°C. After the suspension has dried onto

the surface the temperature is allowed to equilibrate to room temperature after which a small aliquot of the test disinfectant liquid (100µl) is placed on the face of the surface for 5 minutes. The surfaces are then placed in 10ml of neutralizing agent with 5g of glass beads. The suspensions are shaken for 1 minute at 150 rpm and then used for serial dilutions (10^{-3}—10^{-6}) followed by performing the pour plate technique with tryptone soy agar. Plates are incubated at 37°C for 24 hours. However the current CEN method EN13697:2001 [133] does not address the efficacy of disinfectants against biofilm. Due to the short contact time there is little opportunity for cells to initiate biofilm formation before disinfectant is applied. Therefore there are no guidelines to demonstrate the efficacy of disinfectant products against an established biofilm.

4.1.3. The ASTM standards for evaluating disinfectant efficacy

The American Society for Testing and Materials (ASTM) provides guidance and protocols for assessing the efficacy of disinfectants against established bacterial biofilms. There are currently two active standards (published in 2012) in use for examining the efficacy of disinfectants against an established biofilm.

The standard designated E2871-12 describes the method for evaluating efficacy of disinfectants against a *P. aeruginosa* biofilm grown in the CDC biofilm reactor (CBR) using a single tube method [125]. The method uses a similar procedure for cultivating a biofilm on surfaces as outlined in this thesis. The previously published ASTM method for growing the biofilm using this reactor (E2562) has also been published elsewhere [72, 204]. The method used for assessing the disinfectants differs from the method described in this thesis in the volume of disinfectants and neutralizing agents used. The standard protocol (E2871-12)[125] does not outline what

contact time should be used with the disinfectants. Therefore this component should be adjusted based on the specification of the particular disinfectant in use.

The standard designated E2799-12 outlines a method of testing disinfectant efficacy against an established *P. aeruginosa* biofilm using the MBEC assay [126]. The MBEC assay consists of a 96 well microtiter plate with appendages (pegs) attached to the lid. The biofilm is grown on the surface of the pegs in a closed batch system and each well in the plate may contain different test strains or concentration of nutrients. After the biofilm is established, the lid (with pegs attached) can be attached to a new plate with disinfectant agents contained in each well. After a specific contact time, the pegs can be broken off the top of the lid and placed in broth. The broth can be used for serial dilutions and enumeration via the plate count method. The biofilm development time using this method is 24 hours which may be too short to test the disinfectant efficacy against a developed biofilm as depending on the conditions within the system. The time allowed for biofilm development was also discussed previously in more detail in chapter 3. The sonication time was also longer (30±5minutes) than recommended using other systems including the CBR, which may reflect the organisms under examination. There are also no recommendations for specific contact times with disinfectants using the MBEC method.

4.1.4. The influence of biofilm models on the efficacy of disinfectant agents

As discussed, there are a number of different models for examining the efficacy of disinfectant agents against bacterial suspensions, surface adherent bacterial cells and cells associated with a biofilm matrix. The

availability of multiple models and recommendations tends to result in difficulties with the comparability of data from different studies research.

Buckingham-Meyer *et al.* compared 5 different biofilm test methods. This work used identical glass surfaces to provide biofilm substratum and used a standardised biofilm removal and enumeration method throughout (control coupon–treated coupon). The work evaluated biofilm formation of two strains of *P. aeruginosa* and *S. aureus* [117]. The methods varied quite significantly in relation to fluid shear and operation system (batch or continuous flow system). The CDC reactor (as previously described) and a drip flow reactor model (coupons placed in a vertical line in a reactor vessel which comes into contact with a flow of inoculated medium) were used. Other methods included a dehydrated biofilm system (coupons taken out of CDC reactor and dried in accordance to ASTM standards) and the dried surface method (heavy inoculum of test organism allowed dry onto the surface for 2 hours in accordance to the ASTM standards). Buckingham-Meyer suggested that methods such as the dried surface technique and static biofilm method resulted in a ~4 log_{10} reduction in the number of viable cells recovered from the surface after contact with sodium hypochlorite. In contrast, after contact with same disinfectant agent there was only a ~1-2 log_{10} reduction in the number of cells when the biofilm was formed using the CBR method [117]. This work emphasised the difference in the apparent efficacy of disinfectant products used against the same test strains may be greatly influenced by the method applied.

4.1.5. Disinfectants Used

Three disinfectants used for this work sodium hydroxide (1 Mole –M), sodium hypochlorite (500mg/L) and benzalkonium chloride (0.02% volume/volume –v/v). The concentrations are based on review of the

concentrations used in industry and comparable to those used in other work published in this area [113, 116, 134, 205-206].

Sodium hydroxide (molecular formula NaOH) is frequently referred to as caustic soda due to the highly caustic (corrosive) properties of the alkaline base (high pH). Sodium hydroxide is commonly used in the meat processing industry to clean carcasses, surfaces and equipment due to the ability of NaOH to break down lipids including animal fats and organic matter. Previously published research has indicated that *Salmonella* can survive low concentrations of sodium hydroxide [206]. McKee *et al.* isolated *S.* Typhimurium in a container with a solution of 1% sodium hydroxide (0.25M) used for scalding raw meat [206]. *Salmonella* survival in disinfectant solutions may also result in cross contamination onto food. Chorianopoulos *et al.* also demonstrated that *S. enterica* biofilm can survive after contact with a disinfectant product containing of sodium hydroxide [147]. The research presented by Chorianopoulos *et al.* indicated that solution of sodium hydroxide (1mM) only marginally reduced the number of viable cells (0.12-1.50 $\log_{10}$ reduction) recovered from the *S. enterica* biofilm (established over 5 days) after contact for 60 and 180 minutes whereas the use of 100% hydrosol or essential oils and 80% ethanol resulted in a more significant reduction of cells recovered (>4 $\log_{10}$ reduction) [147].

Sodium hypochlorite (molecular formula NaClO) is a membrane-active oxidising compound that permeates and destroys the bacterial cell wall proteins. Sodium hypochlorite is frequently used as a disinfectant agent for hard surfaces and blood spillages [129]. Park *et al.* compared the efficacy of 100ppm (100mg/L) aerosolized sodium hypochlorite and concentration of

peracetic acid (100, 200, 400ppm) for inactivating *E. coli, L. monocytogenes* and *Salmonella* biofilm cells formed on stainless steel and polyvinyl plastic (PVC) over 6 days [207]. Parks *et al.* found that treatment with peracetic acid was more effective than treatment with sodium hypochlorite for the 3 species examined. The results presented by Park *et al.* indicate that exposure of *Salmonella* biofilm to 100mg/L of sodium hypochlorite for 5-50 minutes resulted in a ~1 log_{10} reduction in the number of cells recovered from steel. Exposure of the *Salmonella* biofilm to the same concentration of peracetic acid results in a greater reduction after 50 minutes contact time. However exposure to a higher concentration of peracetic acid (200mg/L) for 10, 30 and 50 minutes resulted in a more significant reduction in the number of *S.* Typhimurium cells recovered from the steel surface (>4 log_{10} reduction). A similar pattern for log_{10} reduction was reported when the PVC surfaces was used as a biofilm substratum [207]. It may have been of use to also investigate if a higher concentration of sodium hypochlorite would also reduce the number of cells recovered from the surface.

Vestby *et al.* used a high working concentration of 0.05% (500mg/L) sodium hypochlorite to investigate the log_{10} reduction of a 48 hour *Salmonella* biofilm cells recovered from glass slides after exposure time of 5 minutes [113]. The results indicated that there was a mean reduction of 2.4 log_{10} of *S. enterica* cells recovered from the surface following treatment for 5 minutes. However, in similar research by the same group Møretrø *et al.* reported a mean log_{10} reduction of 0.5-1.00 in the number of cells recovered from the glass surfaces using the same method, product containing sodium hypochlorite and similar *S.* Agona serovars (both *S.* Agona strains isolated from a fish processing environment) [134].

As previously discussed Buckingham-Meyer *et al.* also used sodium hypochlorite at multiple concentrations (100mg/L, 500mg/L and 1000mg/L) to treat *Pseudomonas* species and Staphylococcal species biofilm on glass surfaces using four biofilm models, including the CBR [117]. Three of the methods used had a biofilm development time of 48 hours and two hours for the dried surface method as described under CEN guidelines EN13697 [117]. The results indicated that exposure to sodium hypochlorite at any of the concentrations against a 48 hour biofilm developed using the CBR only resulted in a ~1-2 $\log_{10}$ reduction. However the use of other methods such as the dried surface biofilm or the static biofilm method generally resulted in a much greater $\log_{10}$ reduction in the number of cells recovered from the surface (2-4 of reduction) [117, 133]. They also indicated that a two-fold increase in concentration of sodium hypochlorite had little to no additional impact [117].

Benzalkonium chloride (molecular formula $C_6H_5CH_2N(CH_3)_2RCl$) is a quaternary ammonium compound synthesized from ammonium chloride (NH_4Cl) [208]. Previous research has reported multiple species including *Serratia* and *Pseudomonas* species had the ability to proliferate in a low concentration benzalkonium chloride solution leading to contamination of the disinfectant product [209-210]. Benzalkonium chloride resistant bacteria have also been linked to increased resistance to antibiotics [16, 136]. Based on the user concentration recommended by the disinfectant manufacturer, Vestby *et al.* used a concentration of 0.02% to investigate if the ability of *Salmonella* strains to persist in the environment was associated with resistance to disinfectants. They reported that a cleaning product with 0.02% benzalkonium chloride only resulted in marginal reduction of cells recovered from the surface (0.8-1 $\log_{10}$ reduction) [113].

As discussed previously, Møretrø *et al.* used the same biofilm model to examine the effect of disinfectants against *S. enterica* biofilm [134]. Møretrø *et al.* found exposure to benzalkonium chloride achieved a ~2 $\log_{10}$ reduction in *S. enterica* numbers recovered from biofilm [134]. This is twice the reduction in the number of cells reported previously by Vestby, however in this instance it is not clear that the same benzalkonium product was used by both researchers therefore the difference in disinfectant product may have contributed to the variation in $\log_{10}$ reduction achieved. The product used by Møretrø *et al.* also included 5-15% acetic acid in the composition [134]. The disinfectant products chosen by Møretrø *et al.* were based on a review of the procedures in place in a number of food processing environments [134].

Wong *et al.* evaluated multiple concentrations of benzalkonium chloride in addition to 5 other disinfectant compounds using the MBEC method [149, 198]. Wong *et al.* reported that benzalkonium chloride was the least effective disinfectant against an established (comparators were citric acid anhydrous, chlorhexidine gluconate, ethanol, quaternary ammonium compound and sodium hypochlorite [149, 198]. In order to achieve complete eradication of viable cells Wong *et al.* used a concentration of 1.5% which is over twice the manufactured recommended concentration. However, there were also a number of inconsistencies with this work, Wong *et al.* found that the number of cells recovered from the surface did not increase over time [149]. Moreover, Wong *et al.* found that concentrations of 1.31, 2.62 and 5.25 g/L of sodium hypochlorite resulted in no cells recovered from the surface. However, ten-fold higher concentrations of sodium hypochlorite (26.25 and 52.5 g/L) resulted in a high population of cells (10^2-10^4) recovered from the surface. Wong *et al.* suggested that the low concentration of hypochlorite and the ratio of acid-to-protein may result in oxidative unfolding and aggregation of proteins[149]. Disruption of cellular proteins in low concentrations of

sodium hypochlorite has also been discussed elsewhere [211]. Nevertheless, the improvement in efficacy of sodium hypochlorite at lower concetrations, as compared with higher concetrations also leads to uncertainty in the concentration of the disinfectant nescessary to elimiate cells from the biofilm.

4.1.6. Neutralizing agents

Neutralizing agents are frequently used in order to validate that the observed $\log_{10}$ reduction in viable cells occurs within the intended contact time. Neutralizing agents should quench the antimicrobial properties of the disinfectant compounds once added and also should not contribute to reducing the number of viable cells. ASTM standard E1054-08 provides guidelines on performing tests to validate the use of selected neutralization agents [212]. The ASTM standards provide guidance on examining neutralizer effectiveness, toxicity, suitability of the test material and viability of the organism through performing suspension tests while altering each variable individually (disinfectant, neutralizer, saline and media). The suspension tests should be followed by inoculating and drying the product onto the test surface and using plate count methods to determine if any of the neutralizing components results in a reduction in numbers of viable cells.

Reichel *et al.* highlighted the importance of storage concentration, temperature and volume of neutralizing agent used. As a result of decreased efficacy after storage, fresh disinfectants and neutralizing agents should be made on each day testing is performed. With the number of disinfectant compounds continually increasing, selection of specific neutralizing agents should reflect the active agents within the disinfectant under examination. CEN standards EN13697:2001[133] and EN1040:2006 [131] list sodium thiosulfate as an appropriate neutralizing agent.

Dey/Engley (Difco) neutralization broth has also been recommended by the ASTM standard E2871-12 [125].

Sodium thiosulfate (chemical formula $Na_2S_2O_3$) is commonly used for neutralizing disinfectants containing iodine or chlorine derivatives [71, 117, 213]. Sodium thiosulfate acts as a neutralizing agent by reacting with the free chlorine in a solution and releasing the components as harmless molecules such as water (H_20) and sodium chloride (NaCl) [214]. Dey/Engley neutralization broth inhibits bacterial growth. Dey/Engley has previously been used to neutralize sodium hydroxide and benzalkonium chloride [134, 149]. Dey/Engley contains a mixture of neutralizing agents including sodium thioglycolate, sodium thiosulfate, sodium bisulfite, polysorbate 80, lecithin (soybean extract) that allows broad spectrum efficacy against disinfectant agents [215].

4.1.7. Use of test controls

The inclusion of test controls (coupons or test surfaces) is crucial in order to validate the efficacy of any antimicrobial or disinfectant under examination. The use of additional coupons (not in contact with disinfectants or neutralizing agent) confirms that any reduction in cells enumerated is independent of any other chemical agents present in the media or other factors such as decline in viable cells over extended time. Hamilton *et al.* also highlighted the importance of using controls in order to validate harvesting and disaggregation steps of biofilm cells for accurate enumeration of any cell reported antimicrobial or disinfectant effect [183]. This is supported by the data presented in earlier chapters of this thesis demonstrating that completeness of biofilm removal from surfaces is dependent on strain and the age of the biofilm.

4.1.8. Duration of contact time

According to the CEN standards a contact time between the bacterial suspension and the test disinfectant should not be more than 5 minutes. However the CEN standards only examine efficacy of disinfectant products against surface adherent bacteria. The time allowed for disinfectant agents in contact with biofilm may be a crucial component contributing to complete destruction of the biofilm cells. Møretrø used a contact time of 5 minutes for the biofilm and suspension assay [134]. Gehan *et al.* used contact times of 10, 30 and 60 minutes in order to assess the efficacy of disinfectants in a suspension test with a set of organisms (*P. aeruginosa, E. coli, S. aureus, S. Typhimurium, A. fumigates, Fusarium* spp.) against quaternary ammonium compound and sodium chloride [216]. Parks *et al.* found an increased contact time (50 minutes) was necessary to significantly reduce the number of viable cells of *S*. Typhimurium attached to stainless steel and polyvinyl plastic surfaces using aerosolized sodium hypochlorite [207]. Nguyen *et al.,* used contact times of 0.5, 1, 2, 3, 5, and 7 minutes to evaluate the efficacy of quaternary ammonium compounds, sodium hypochlorite and a solution of peroxyacetic acid/organic acids *S*. Typhimurium biofilm [217]. Therefore, in order to gain a better insight into the time needed for effective disinfection of an established biofilm, contact times of 10, 45 and 90 minutes were evaluated in this work.

4.1.9. Survival of *Salmonella* cells after contact with disinfectant agents.

Although the accepted criteria for disinfectant efficacy are defined in terms of quantitative $\log_{10}$ reduction in cell counts in practical terms the persistence of even very low residual numbers of viable *Salmonella* is likely to result in regrowth over time given appropriate conditions. Therefore a very marked reduction in mean $\log_{10}$ density or even reduction below the level of enumeration is not sufficient to ensure that the environment is *Salmonella* free. To assess if biofilm eradication was complete (if

sterilisation was achieved) it was considered that following disinfectant exposure, in addition to determination of biofilm density by quantitative methods an entire coupon with attached biofilm should be immersed in broth to determine if the coupon had been sterilised. Mangalappalli-Illathu and Korber indicated that *Salmonella* biofilm exposed to sub-lethal concentrations of benzalkonium chloride over 144 hours (1 µg/ml continuously or 10µg/ml daily) resulted in *Salmonella* survival and re-growth following exposure to a higher concentration of benzalkonium chloride (500µg/ml for 10 minutes) [106]. Bacterial re-growth post disinfection treatment was assessed through measuring biofilm thickness and examining the molecular and proteomic properties of the biofilm before and after treatment. The authors found that biofilm (continuously exposed to benzalkonium chloride) continued to increase in thickness more than biofilm that was not previously exposed to benzalkonium chloride. However, the thickness of all 168 hour biofilm continued to grow for over 24 hours after exposure to benzalkonium chloride [106]. Previous authors have also found that *Salmonella* cells that survive disinfectant treatment also exhibit resistance to a number of disinfectant and antimicrobial agents [132].

Table 4.1: Sub-set of strains used to examine the efficacy of disinfectant against an established biofilm

Strain	Strain Number
Salmonella enterica subspecies *enterica* serovar Agona	SL483
Salmonella enterica subspecies *enterica* serovar Agona	S09-0494
Salmonella enterica subspecies *enterica* serovar Typhimurium	SL1344
Salmonella enterica subspecies *enterica* serovar Enteritidis	S09-0717

The origin and details of each strain in this subset was previously provided in table 2.1 in chapter 2.

4.2. Methods

4.2.1. Broth Minimum Inhibitory Concentration Method

I. The test organisms were stored at -20°C on glycerol protect beads between experiments.

II. All 13 strains described in chapter 2 were used to establish the MIC.

III. Before each experiment the test strain was defrosted on the bench and streaked on Tryptic Soy Agar (TSA) and Xylose Lysine Deoxychocolate (XLD) plates which were incubated for 24 hours at 37°C.

IV. The three disinfectants were made to above the working concentration on each day of experiment in order to assess a range of concentrations. Sodium hypochlorite (2000mg/L), sodium hydroxide (4M) and benzalkonium chloride (0.08%) solution were prepared using sterile H_20 and filter sterilized compounds.

V. Doubling dilutions were performed to achieve the test concentrations.

VI. Concentrations assessed were

- Sodium hypochlorite (1000mg/L, 500mg/L, 250mg/L, 125mg/L, 75mg/L)
- Sodium hydroxide (2M, 1M, 0.5M, 0.25M, 0.125M)

VII. Benzalkonium chloride (0.04%, 0.02%, 0.01%, 0.005%, 0.0025%)Isolated colonies were picked off the TSA plate and made to a 0.5 McFarland suspension (~1.5-5x10^8 CFU/ml) in sterile water and gently shaken.

VIII. 1ml of the suspension was added to 9ml of sterile Tryptic Soy Broth (TSB) and vortexed vigorously.

IX. Aliquots of 100µl of the bacterial suspension (~1.5-5x10^7 CFU/ml) were placed into each well of a 96 well microtitre plate using a pipette.

X. Aliquots of 100µl of each disinfectant at the primary concentrations were placed in each of the corresponding wells.

XI. Therefore 100µl of 2000mg/L of sodium hypochlorite and 100 µl of broth resulted in a working concentration of 1000mg/L of sodium hypochlorite.

XII. The plates were incubated for 24 hours at 37°C.

XIII. The plates were examined visually to assess turbidity after incubation, with the presence of pellicle formation indicative of bacterial growth.

XIV. Each disinfectant MIC test was performed in triplicate wells for each strain and each test was performed on 2 different days.

4.2.2. Disinfectant testing method using the CBR

I. Twenty-four concrete coupons were used as a substratum for biofilm growth in each biofilm reactor. A standard biofilm (48 hour biofilm growth) was achieved by following the method described in chapter 2 (Section 2.5.1) the older biofilm (7days- 168 hours) was achieved as described in chapter 3 (Section 3.2).

II. After biofilm formation was complete the following method was used.

III. Fresh solutions of disinfectants were used for each experiment.

IV. Sodium hypochlorite (500mg/L), sodium hydroxide (1M) and benzalkonium chloride (0.02%) were made using sterile H_20 and filter sterilization.

V. The coupons were released from the CDC biofilm reactor and aseptically placed into individual wells of a 24 well microtiter plate.

VI. After 10, 45 and 90 minutes contact time with 1ml of each of the disinfectant at the test concentration the solution was aspirated out of the plate and the coupons were then immersed in 1ml of the appropriate neutralizing agent for 30 minutes (see Figure 4.1).

VII. Difco™ D/E Neutralizing broth (39g/L) (Becton Dickenson) was used to neutralize sodium hydroxide and benzalkonium chloride and sodium thiosulfate (11.2g/L) to neutralize sodium hypochlorite.

VIII. The coupons were separated into the groups and assessed for cell reduction and cell recovery.
- Cell Reduction
- Cell Recovery

IX. The 1st group (cell reduction) of coupons were aseptically placed in capped glass universal containers with 10ml of PBS and sonicated using the conditions outlined in chapter 2 (section 2.5.1).

X. The universals (containing the coupon) were vortexed at high speed to break down biofilm clumps and serial dilutions were made of the PBS solution (coupons were taken out of the glass containers for method described in chapter 2 as vortexing the surface in glass containers was not possible for steel or glass coupons).

XI. The plate count method was performed as previously described in chapter 2 (section 2.5.1)

XII. The Mean Log_{10} Density was used to calculate the Log_{10} reduction.

XIII. Log_{10} reduction = Mean Log_{10} untreated control coupons–Mean Log_{10} treated coupons as previously described (ASTM Standard E2871-12) [125].

XIV. Each run performed twice using 1 coupon for each contact time and disinfectant from each run.

XV. The suspended biofilm solution was serially diluted and spread onto 3 replicate TSA plates using the plate count technique previously described.

XVI. The 2nd group (cell recovery) coupons were aseptically placed in capped glass universal containers with 10ml of TSB.

XVII. The coupons in the universals were incubated for 37°C for 24 hours.

XVIII. A change in the turbidity of the broth was used to verify the presence of viable cells on the surface.

XIX. Turbid broths were subcultured to confirm that turbidity was related to re-growth of *Salmonella* (based on typical morphology).

XX. A set of coupons were also treated with sterile water (Control Coupon) and with the two neutralizing agents to ensure that the neutralizing agent was not reducing the number of culturable cells.

Table 4.2: Minimum Inhibitory Concentration of sodium hydroxide, benzalkonium chloride and sodium hypochlorite.

Strain No:	Strain	*N*	**Sodium Hydroxide** Moles per Litre (M)	**Sodium Hypochlorite** micrograms per Litre (mg/L)	**Benzalkonium Chloride** Percentage volume (%v/v)
27853	*P. aeruginosa*	9	0.25	125	0.005
S08-0601	*S.* Agona	9	0.25	125	0.005
S09-0494	***S.* Agona**	**9**	0.25	125	0.005
S09-0046	*S.* Agona	9	0.25	125	0.005
S09-0371	*S.* Agona	9	0.25	125	0.005
S09-0479	*S.* Agona	9	<0.125	<75	0.005
SL483	***S.* Agona**	**9**	0.25	125	0.005
S09-0419	*S.* Typhimurium	9	0.25	125	0.005
S08-0408	*S.* Typhimurium	9	0.25	125	0.005
SL1344	***S.* Typhimurium**	**9**	0.25	125	0.005
PB449/LT2	*S.* Typhimurium	9	0.25	125	0.005
S09-0717	***S.* Enteritidis**	**9**	0.25	125	0.005
S09-0004	*S.* Enteritidis	9	<0.125	<75	0.005

Table 4.2 displays the Minimum Inhibitory Concentration (MIC) of disinfectants required to inhibit bacterial cell growth in a fresh growth medium (TSB) incubated for 24 hours at 37°C. Each strain was tested in three replicate wells on 1 plate for each experiment. The strains include 1 *Pseudomonas aeruginosa,* 6 *Salmonella enterica subspecies enterica serovar* Agona strains, 6 *S.* Typhimurium strains and 2 *S.* Enteritidis strains. The strains highlighted in bold are the strains that were used in biofilm studies further described in this chapter.

4.3. Results

4.3.1. Suspension test to establishing Minimum Inhibitory Concentration (MIC)

Table 4.2 displays the results of the MIC assay which indicates that a concentration of 0.5M (sodium hydroxide), 250mg/L (sodium hypochlorite) and 0.01% (benzalkonium chloride) were sufficient to inhibit all bacterial growth after incubation with the bacterial suspension for 24 hours at 37°C. The same MIC was reached across all 4 strains used for biofilm research in this chapter (highlighted in bold). Two of the thirteen strains (S09-0471 and S09-0004) had a lower MIC.

4.3.2. Assessing the efficacy of three disinfectants against an established 48 hour biofilm

Table 4.3 demonstrates the mean $\log_{10}$ density of cells recovered from a biofilm (without contact with any disinfectant agent—control coupons) and the mean $\log_{10}$ density of cells recovered from a biofilm after contact with sodium hydroxide (1M), sodium hypochlorite (500mg/L) and benzalkonium chloride (0.02%) for 10-, 45- and 90-minutes.

There was very little difference between the mean $\log_{10}$ density of cells recovered from the *S*. Agona SL483 biofilm and the *S*. Typhimurium SL1344 biofilm not in contact with the disinfectant (control coupons). Treatment with sodium hydroxide reduced the number of viable cells recovered (for both *S*. Agona SL483 and *S*. Typhimurium SL1344 biofilms). After exposure for 10-, 45- and 90-minutes with sodium hydroxide no cells were recovered from the 48 hour biofilm. Therefore the mean $\log_{10}$ reduction after contact with sodium hydroxide was 7.63 CFU/coupon (strain SL1344) and 7.66 CFU/coupon (strain SL483). In addition, no cells were recovered when

coupons were incubated for 24 hours in TSB at 37°C after contact with sodium hydroxide (10-, 45- or 90-minutes) and neutralized as described. Thus sodium hydroxide effectively sterilised the coupon with the 48 hour biofilm.

However, as illustrated in Table 4.3 sodium hypochlorite (500mg/L) and benzalkonium chloride (0.02%) had little impact on reducing the number of viable cells recovered after a contact time of up to 90-minutes. The mean $\log_{10}$ density of cells recovered from the *S*. Agona SL483 biofilm was only marginally less after 10-, 45- or 90-minute of exposure. Following treatment of the *S*. Typhimurium SL483 biofilm with sodium hypochlorite there was just over 1 $\log_{10}$ reduction in cells recovered from the surface and less than 1 $\log_{10}$ reduction in the number of cells recovered was achieved after 90 minutes treatment with benzalkonium chloride. Similarly, there was only a marginal reduction in the number of cells recovered from the *S*. Agona SL483 biofilm after contact with sodium hypochlorite and benzalkonium chloride after contact for 10-, 45- and 90-minutes. As expected there was dense re-growth in the TSB broth in which a whole treated coupon was placed. For both strains there was an apparent impact of time of exposure to sodium hypochlorite and benzalkonium chloride in that there was a gradual reduction in biofilm density following 10 to 45 to 90 minutes of exposure.

Table 4.3: Mean log_{10} density of cells recovered from the 48 hour biofilm after contact with disinfectants (measured in Mean Log_{10} CFU/coupon)

Disinfectant	*N*	Time	48 Hour biofilm –direct spread plate results					
			Strain 1 - SL1344			Strain 2 - SL483		
			Mean	LR	SD	**Mean**	LR	SD
Disinfectant free Control			**7.63**		0.02	**7.66**		0.01
Sodium Hydroxide 1 Mole	6	10	**0**	7.63	0	**0**	7.66	0
	6	45	**0**	7.63	0	**0**	7.66	0
	6	90	**0**	7.63	0	**0**	7.66	0
Sodium Hypochlorite 500mg/L	6	10	**7.48**	0.15	0.03	**7.53**	0.13	0.13
	6	45	**7.45**	0.18	0.04	**7.36**	0.30	0.25
	6	90	**6.52**	1.11	0.19	**7.15**	0.51	0.36
Benzalkonium Chloride 0.02%	6	10	**7.41**	0.22	0.09	**7.48**	0.18	0.16
	6	45	**7.32**	0.31	0.01	**7.24**	0.42	0.34
	6	90	**6.80**	0.83	0.60	**6.69**	0.97	0.18

Table 4.3: Presents mean log_{10} density of viable cells recovered from *Salmonella enterica* serovar *enterica* subspecies Typhimurium SL1344 and *S*. Agona SL483 biofilm attached to concrete coupons over 48 hours. After contact with 3 disinfectants for 10-, 45- and 90 minutes the viable cells were recovered and enumerated using the spread plate technique on three replicate TSA plates. Each experiment was repeated twice. Table 4.3 presents the mean log_{10} density (CFU/coupon) and the standard deviation of all 6 counts. The log_{10} reduction (LR) was calculated by subtracting mean log_{10} density of cells from the test coupon from the mean log_{10} density of cells recovered from the control coupon i.e. control coupon – test coupon.

4.3.3. Assessing the efficacy of disinfectants against an established 168 hour biofilm

Table 4.4 summarizes the mean log_{10} density of cells recovered from the concrete test surfaces after the development of a biofilm over an extended period of time (7 days-168 hours). The mean log_{10} density of viable cells was assessed for four *Salmonella* strains- *S*. Typhimurium strains SL1344, *S*. Agona SL483, *S*. Agona outbreak related strain S09-0494 and *S*. Enteritidis strain S09-0717. There were limited differences between the 4 strains when grown for 168 hours on the concrete surfaces.

The mean log_{10} density of cells recovered from the *S*. Typhimurium SL1344 was 9.74 CFU/coupon. After contact with sodium hydroxide for 10 minutes there was a ~1.5 log_{10} reduction in the number of cells recovered (8.24CFU/coupon). After 90 minutes there was an average of a 2 log_{10} reduction in the number of cells recovered from the SL1344 biofilm. This trend was similar for the two *S*. Agona strains SL483 and S09-0494. However, sodium hydroxide was slightly less effective for reducing the number of cells recovered from the *S*. Enteritidis biofilm (< 1 log_{10} reduction).

Sodium hypochlorite was less effective at reducing the number of cells than sodium hydroxide. The mean log_{10} density of cells recovered from the *S*. Typhimurium SL1344 biofilm after 10 minutes exposure was 9.48 CFU/coupon and was only marginally reduced to 9.40 CFU/coupon after 90 minutes exposure which indicates there was ≤0.2 log_{10} reduction after extensive contact with the disinfectant. This suggests that increased time of exposure appeared to have a limited effect.

Benzalkonium chloride was the least effective disinfectant against an established biofilm using all 4 strains examined. However the difference between the number of cells recovered after treatment with benzalkonium chloride and sodium hypochlorite was marginal in most instances therefore may likely be of no practical significance. After 90 minutes contact with benzalkonium chloride the number of cells recovered from the surface was only marginally reduced by less than 0.30 log_{10} CFU/coupon.

Table 4.4: Mean log_{10} density of viable 168 hour biofilm cells after contact with disinfectants (measured in Mean Log_{10} CFU/coupon)

			SL1344			SL483			S09-0494			S09-0717		
			Mean	LR	SD	**Mean**	LR	SD	**Mean**	**LR**	SD	**Mean**	LR	SD
Disinfectant free Control	*N*	Time	**9.74**		0.04	**9.78**		0.04	**9.74**		0.03	**9.68**		0.01
Sodium Hydroxide 1 Mole	6	10	**8.24**	1.50	0.24	**8.13**	1.65	0.38	**8.10**	1.64	0.36	**8.89**	0.79	0.19
	6	45	**8.01**	1.73	0.50	**7.96**	1.82	0.33	**8.13**	1.61	0.21	**8.88**	0.80	0.16
	6	90	**7.72**	2.02	0.57	**7.45**	2.33	0.12	**7.74**	2.00	0.26	**8.84**	0.84	0.17
Sodium Hypochlorite 500mg/L	6	10	**9.48**	0.26	0.02	**9.56**	0.22	0.13	**9.32**	0.42	0.01	**9.33**	0.35	0.01
	6	45	**9.43**	0.31	0.07	**9.51**	0.27	0.13	**9.28**	0.46	0.07	**9.00**	0.68	0.25
	6	90	**9.40**	0.34	0.08	**9.50**	0.28	0.14	**8.77**	0.97	0.74	**8.89**	0.79	0.32
Benzalkonium Chloride 0.02%	6	10	**9.68**	0.06	0.08	**9.74**	0.04	0.05	**9.71**	0.03	0.06	**9.58**	0.10	0.07
	6	45	**9.65**	0.09	0.09	**9.75**	0.03	0.04	**9.69**	0.05	0.07	**9.51**	0.17	0.09
	6	90	**9.64**	0.10	0.09	**9.47**	0.31	0.24	**9.65**	0.09	0.12	**9.49**	0.19	0.09

Table 4.4: Presents mean log_{10} density of cells recovered from concrete surfaces after contact with disinfectant agents. Strains examined incorporated *Salmonella enterica* subspecies *enterica* serovar Typhimurium SL1344, *Salmonella* Agona SL483, *S*. Agona S09-0494 and *Salmonella* Enteritidis S09-0717. After contact with 3 disinfectants for 10-, 45- and 90 minutes the viable cells were recovered and enumerated using the spread plate technique on three replicate TSA plates. Each experiment was repeated twice. Table 4.2 presents the mean log_{10} density (CFU/coupon) and the standard deviation (SD) of all 6 counts. The log_{10} reduction (LR) was calculated by subtracting mean log_{10} density of cells from the test coupon from the mean log_{10} density of cells recovered from the control coupon i.e. control coupon – test coupon.

4.4. Discussion

Suspension tests were performed in order to determine the minimum inhibitory concentration (MIC) of the disinfectant agents. The MIC is used to determine the concentration of disinfectants that inhibits visible bacterial growth. The purpose of this was to establish the efficacy of the disinfectants chosen against the strains studied in planktonic form. The results of the suspensions tests described Table 4.2 indicate that concentrations of 250mg/L (sodium hypochlorite), 0.5M (sodium hydroxide) and 0.01% (benzalkonium chloride) were sufficient to inhibit visible bacterial growth. If disinfectant products are examined solely using a suspension test method such as the ASTM standard 1040 [131] it is possible that while bacterial cells may be inhibited using low concentration of disinfectant agents the agents may not be effective if the bacterial cells have formed a biofilm.

Notwithstanding the susceptibility of the strain in planktonic form, sodium hypochlorite and benzalkonium chloride (at a concentration twofold higher than the MIC) were not effective at eliminating all biofilm developed after 48 hours period and none of the 3 agents effective in eliminating the 168 hour biofilm (SL1344 and SL483) after contact times of 10, 45 and 90 minutes. Møretrø *et al.* also reported that the MIC of disinfectant agents such as 0.01-0.005% benzalkonium chloride was effective at eliminating bacterial growth in a suspension test however the same concentration was not effective at eliminating all bacterial cells from an established biofilm [134].

4.4.1. The log_{10} reduction achieved by sodium hydroxide

Sodium hydroxide was the most effective disinfectant of all 3 examined. When used to treat a 48 hour biofilm no viable cells were detected after 10

minutes of contact. In addition no cells were recovered when the intact biofilm was incubated in broth for 24 hours confirming that the coupon had been sterilised. However the same concentration of sodium hydroxide was not effective at eliminating cells when a biofilm was established over an extended period of time. The results presented in this chapter indicate that despite an extensive contact time of 90 minutes only achieved a 2 log_{10} reduction in the number of cells recovered from the surface. There are limited studies performed on sodium hydroxide as a disinfectant against an established *Salmonella* biofilm although its use has been documented in industry [218]. Chorianopoulos *et al.* used a 1mM solution of sodium hydroxide as part of an evaluation of the efficacy of eight disinfectants against *Salmonella* Enteritidis [147]. After treatment of a 5 day biofilm for 60 and 180 minutes Chorianopoulos *et al.* found that the log_{10} reduction of *S.* Enteritidis cells recovered from the biofilm post treatment was with sodium hydroxide was 0.12 and 1.50 after 60 and 180 minutes respectively. This trend (slight reduction over the extended period of time) corresponds with what was reported in this chapter although the log_{10} reduction reported by Chorianopoulos *et al.* was lower [147]. This difference in log_{10} reduction may reflect variation in biofilm development methods and concentration of the disinfectant agent used. The work described by Chorianopoulos *et al.* examined the efficacy of sodium hydroxide at concentrations of 1mM, whereas the work in this chapter used a 1M solution based on the results of the MIC test.

As discussed previously, there are very limited studies examining the efficacy of sodium hydroxide against an established biofilm. However Parkar *et al.* also found that a 2% concentration of sodium hydroxide (0.5M) with nitric acid (HNO_3) was the most effective disinfectant at reducing the number of thermophilic *Bacillus flavothermus* cells attached to steel after 18 hours biofilm formation [219]. The authors indicated that 30 minutes contact with the sodium hydroxide mixture at 75°C was

effective at eliminating all viable cells from the surface (7 $\log_{10}$ reduction) [219]. However cells were recovered from the biofilm (5 $\log_{10}$ reduction) if the cleaning temperature was reduced to 60°C or if the concentration was reduced to 0.25M. This may have serious implications for the food processing industry as the research presented in this chapter demonstrates that cells can continues to form a dense biofilm over an extended period of time and that this biofilm is more tolerant of disinfectant agents.

4.4.2. The $\log_{10}$ reduction achieved by sodium hypochlorite

The research presented in this chapter indicates that sodium hypochlorite inhibited cell growth using a suspension test method. However, viable cells were recovered from a 48 hour and 168 biofilm after contact times of 10, 45 and 90 minutes. In most instances there was less than a 1 $\log_{10}$ reduction after extensive contact (90 minutes) with the biofilm.

Møretrø *et al.* also found that sodium hypochlorite did not eliminate all cells from a *S. enterica* biofilm (incorporating *S.* Agona and *S.* Typhimurium) formed on glass slides after 48 hours [134]. Møretrø and colleagues reported 0.5-1 $\log_{10}$ reduction in the number of viable cells after contact with sodium hypochlorite for 5 minutes. This broadly correlates with the findings presented in this chapter, which also found that sodium hypochlorite was not effective at reducing the number of cells recovered from the surface. However the $\log_{10}$ reduction of cells recovered from both the 48 hour and 168 hour biofilm achieved in this research was lower in most instances (~0.2-0.4 $\log_{10}$ for the 48 hour biofilm) than what was reported by Møretrø *et al.* [134]. Buckingham-Meyer *et al.* also found the use of the CBR to produce a biofilm may resulting in a lower reduction after treatment with disinfectants in comparison to other biofilm development models which was previously discussed in section 4.5 has also been

reported elsewhere has also been which may differences in the biofilm model, as previously discussed in section 4.1.4.

However, in similar research to what was reported by Møretrø *et al.* the same research group reported a ~2.4 log_{10} reduction in the number of cells when using the same sodium hypochlorite containing product on a *S.* Agona biofilm formed on a glass slide over 48 hour [113]. Therefore the difference in log_{10} reduction may be due to strain differences or other changes in conditions between experiments. Møretrø *et al*. examined efficacy of disinfectants against *S*. Agona strain (71-3) taken from feed or fish meal processing environment and an S. Typhimurium ATCC reference strain 14280. Vestby *et al.* used a *S*. Agona strain 71-4 from a similar environment (feed or fish meal processing environment) to determine the log_{10} reduction achieved after contact with an established biofilm on glass slides [113]. Nevertheless, both results reported by Vestby *et al.* and Møretrø *et al.* indicate that sodium hypochlorite did not reduce the number of viable cells below the 4log_{10} threshold required to confirm the efficacy of the disinfectant according to accepted standards (EN13697:2001)[133] which was discussed in detail in section 4.1.2. This finding correlates with what has been found in the results report in this chapter that indicated the same concentration of sodium hypochlorite (500mg/L of sodium hypochlorite which can also be denoted as 0.05%) was not effective against an established biofilm. Tondo *et al.* reported 2-3 log_{10} reduction in *Salmonella* cells recovered from stainless steel and polyethelene surfaces after contact with sodium hypochlorite for 15, 30 and 60 minutes despite the use of high concentration of sodium hypochlorite (up to as high as 800mg/Kg - equivalent to 800mg/L) [220]. The greater log_{10} reduction reported by Tondo *et al.* may have been achieved as a result of a higher concentration of sodium hypochlorite as the research described in this chapter used a concentration of 500 mg/L.

Surprisingly, Nguyen *et al.* found that 50 ppm sodium chlorite (equivalent to 50mg/L or 0.005%) was an effective disinfectant which achieved an >8 log_{10} reduction in the number of viable cells removed from stainless steel and acrylic surfaces against a 24 hour *S*. Typhimurium. A ≥4 log_{10} reduction in cells was achieved against a 48- and 96- hours biofilm on an acrylic surface using a petri-dish with a bacterial suspension in order to achieve biofilm development. As a result, the extensive differences in biofilm model may provide reason for the vast difference in results achieved [217]
.

Ramesh and colleagues also found that 250ppm (250mg/L) sodium hypochlorite was an effective disinfectant which resulted in a mean log_{10} reduction of 6.26 after 1 minute contact time with a *Salmonella* biofilm formed on galvanised stainless steel over 4 days (96 hours) [150]. Ramesh *et al.* reported that after 2 minutes exposure 2 of the 13 disinfectants tested resulted no cells detected recovered from the surface. The two successful disinfectants were a 0.05% concentration of sodium hypochlorite (500mg/L or 500ppm) and a 1% concentration of alkaline peroxide [150].This is in complete contrast the results of this chapter that found even after a contact time of 90 minutes the same concentration was not effective at reducing the number of biofilm cells from the 48 hour or 168 hour biofilm. The large difference in log_{10} reduction may be due to dissimilar biofilm conditions, substratum or biofilm removal methods. Ramesh *et al.* used a static biofilm model (microtiter plate) with stainless steel coupons and surface swabbing for bacterial removal. However, the use of swabbing or scraping may not be an accurate representation of a meaningful log_{10} reduction unless it is confirmed that all viable cells are removed from the surface. SEM analysis was performed to visualise the intact biofilm, however the author gave no indication that SEM was used to confirm complete removal of the treated biofilm. Moreover, some disinfectants may contain other compounds such as gluteraldehyde which

may prevent removal of the bacteria from the surfaces via swabbing, despite the cells remaining viable [221]. Therefore, it is possible that the use of disinfectant products with more than one active agent may result in an under estimation of viable cells remaining [221]. In addition, the high log_{10} reduction of cells may also be a reflection of the biofilm substratum. As demonstrated in chapter one and two of this thesis, concrete is associated with a more dense biofilm compared with other surfaces such as stainless steel. The propensity to support dense biofilm formation may be due to increased surface roughness which may also increase resistance to disinfectant agents.

Joseph *et al.* assessed the log_{10} reduction in viable *Salmonella* cells in biofilm (48 hour development) after treatment with sodium hypochlorite (10, 20, 50 and 100 ppm) and iodophor for 5 intervals of 5-35 minutes [71]. The results from the research suggested that inactivation of *S*. Weltevreden biofilm with hypochlorite (no cells detected) was faster on cement and steel than on plastic (15 min vs. 20 minutes). Joseph *et al*. also reported that exposure for 20 minutes to 100ppm to hypochlorite (100mg/L or 0.01% solution) resulted in no viable cells detected [71]. This does not correlate with the findings reported in this chapter. However, the use of cotton swabs to remove the biofilm was used in this research without providing details of confirmation of complete removal via microscopy or other methods may cast some doubt on the confidence that can be placed in their conclusions.

4.4.3. The log_{10} reduction achieved by benzalkonium chloride

The results presented in this chapter indicated that benzalkonium chloride was the least effective disinfectant against a 48 hour and 7 day *Salmonella* biofilm. The most substantial reduction (1 log_{10} reduction) was achieved

though a contact time of 90 minutes with a 48 hour biofilm. As previously discussed, Wong *et al.* also found that benzalkonium chloride was the least effective disinfectant against an established biofilm in comparison to 5 other disinfectants [149]. In order to achieve complete eradication of viable cells, Wong *et al.* used a concentration of 1.5% which is over twice the manufactures recommended concentration [149]. Vestby *et al.* reported that the use of 0.02% benzalkonium chloride for 5 minutes against a *Salmonella* biofilm formed after 48 hours resulted in a 1-2 log_{10} reduction in the number of cells recovered from the surface [113]. However the log_{10} reduction achieved by Vestby *et al.* is notably higher than the log_{10} reduction described in this chapter despite the use of a 90 minutes contact time. As mentioned previously, the biofilm substratum influences the level of biofilm attachment therefore may also have an effect on the level of biofilm disinfection.

4.4.4. The change in tolerance over an extended period of time

The work presented in this chapter demonstrates that a *Salmonella* biofilm developed over an extended period of time is less vulnerable to the effect of disinfectants products. The results indicate more viable cells were recovered from a 168 hour biofilm than a 48 hour biofilm post treatment with disinfectants. Moreover, products such as sodium hydroxide which is effective using a 48 hour biofilm development time are no longer effective over a biofilm formed over a longer time. Preliminary research performed by Møretrø and colleagues suggested that the mean log_{10} density of a *Salmonella* biofilm on a glass surface did not increase over an extended period of time (9 days) [134]. Moreover, the results of the work indicated that the log_{10} reduction after treatment with sodium chlorite was similar and not dependent on the age of the biofilm, when biofilm was formed over 2, 3, 6 and 9 days biofilm [134]. The suggestion that log_{10} reduction is not affected by the age of the biofilm is in complete contrast to the work

presented in this thesis that found that the density of biofilm cells continued to grow up to 168 hour, as discussed in chapter 3. Moreover, the work presented in this chapter also demonstrated that the age of a biofilm had an important impact on the log_{10} reduction achieved by all three disinfectants.

Using the Calagary biofilm device (MBEC^{TM}), Wong *et al.* reported that the age of a *Salmonella* biofilm did not result in increased resistance to disinfectants. Wong *et al.,* found that in most cases there was a larger log_{10} reduction in cells after contact with disinfectants in the 7- day biofilm when compared to a 3- and 5- day biofilm [149]. However, as previously discussed in chapter 3, the model used by Wong *et al.* to study biofilm growth studied may not support extended biofilm growth possibly due to a smaller surface area available. Therefore, it may be possible that the extent of biofilm reduction may not reflect conditions found in real life scenarios where biofilm cells continued to grow over a longer period of time. This may have serious implications for reporting the level of activity of antimicrobial agents. It is also important to note that in most instances the concentration necessary to reduce the cell numbers above the $>4log_{10}$ threshold was also above the concentration recommended by the manufacturer.

4.4.5. Variability in methods

The lack of standardization of methodology for most biofilm research results in difficulty to compare studies. As demonstrated in the discussion of the results in this chapter, there is limited comparability between this research and previous research due in a large part to variation in methods. Moreover, there is evidence of limited comparability of some research reported using the same research methods, disinfectants and similar strains within a single research group [113, 134]. The ASTM standards

provide a more standardised approach to examining the efficacy of disinfectants against an established biofilm using the CBR (E2871-12) and MBEC (E2799-12) biofilm development methods. However sonication conditions used with the CBR method (are 3 cycles of sonication for 30 seconds with intermittent vortexing) are unlikely to achieve complete removal of all biofilm based on data presented here. Other parameters such as test organism, temperature, pH, and nutrient flow and organic content should also be considered. Moreover, as the publication of these standards is relatively recent, there is relatively little experience with their use and it is not clear how widely they will be adopted due to the high costs associated with the use of the equipment. Other factors such as the method used to establish a biofilm may also influence the efficacy of test products against microbial biofilms of the same strains [117, 134].

4.5. Summary

The research presented in this chapter indicates that there are a number of inconsistencies in the previously published literature in this area. The method chosen for biofilm formation may have a relationship with the amount of biofilm formed on a surface and the resistance of the biofilm to disinfectant treatment. Although standards are emerging the standards have significant limitations such as the time allocated for biofilm development and it is not clear how widely adopted they will be. Moreover, there is evidence presented in this chapter to suggest that the current CEN standards for examining the efficacy of disinfectant products in broth dilutions such as EN1040 may be of limited value, if the bacteria have formed a biofilm on the surfaces.

Secondly, most other research did not achieve a 4 log_{10} reduction in the number of cells recovered from an established biofilm this was also found in most instances in this work -with the exception of sodium hydroxide against a 48 hour biofilm. However the log_{10} reduction after contact with the disinfectants presented in this work was in most cases lower than what has been reported elsewhere. This variation may be a reflection on the differences between methods, disinfectants and strains. This is a considerable uncertainty as to which model best reflects actual conditions in the food processing environment although there is a view that the CBR may provide a more robust method for assessing the ability of *Salmonella* to survive disinfectant treatment compared with other methods.

Furthermore, laboratory studies of disinfectants used for control of *Salmonella* biofilm which focus on standard-48 hour biofilm may have limited applicability in food processing environments. As a result, the focus of the disinfectant studies against an established biofilm changed to 168

hours instead of the standardised 48 hour biofilm in order to investigate the impact of time on bacterial resistance to disinfectant treatment. As demonstrated in this research, it is also important to consider biofilm development over an extended period of time.

The work presented in this chapter indicates that sodium hydroxide was effective at eradicating a standard 48 hour *Salmonella* biofilm however; the same concentration of sodium hydroxide was not effective at eradicating all viable cells. This finding also highlights the importance of examining the efficacy of disinfectant agents against a biofilm formed over an extensive period of time.

Although sodium hypochlorite and benzalkonium chloride resulted in a reduction of viable cells directly after exposure neither of the disinfectants was effective at eliminating all viable cells from the 48 hour or 168 hour biofilm. Moreover, the cells continued to grow after contact with the two disinfectants for up to 90 minutes. As a results studies should also investigate if any remaining cells may be able to recover and grow after treatment. This emphasizes the need for complete elimination of all viable cells from surfaces as opposed to a 4 or 5 log_{10} reduction as it is otherwise possible for cells to continue to grow on the surfaces.

All of these findings may be of critical significance to the food industry, as the results demonstrate the importance of preventing *Salmonella* from becoming established on a food producing campus, as once established in a biofilm, *Salmonella* may be extremely difficult to eradicate.

Chapter 5

Examining *S. enterica* biofilm formation using the microtitre plate based method

5. Abstract

Examining biofilm density on food contact surfaces, as described in Chapters 2 to 4 is time consuming and expensive. Consequently, studies of this kind are frequently limited to a small number of strains of particular interest. As a result, a number of lower cost and high throughput methods for studying biofilm properties have been described. Screening to identify strains with potential to form a dense biofilm by these high throughput methods is frequently used as a preliminary step in biofilm studies. In this chapter, two of these methods are applied to the strains to assess if the different approaches give comparable or complementary results.

Growth of all strains studied on culture media (congo red agar) indicated that all strains displayed the red, dry and rough morphology which is said to be consistent with dense biofilm formers. Using the microtitre plate assay, the data suggests that *S*. Agona strains generally formed a more dense biofilm than *S*. Typhimurium and *S*. Enteritidis at room temperature. However this trend was less apparent when the biofilm was formed at 37°C. In most instances, the density of biofilm as assessed in the microtitre plate did not increase over the extended period of time at room temperature. However, when the biofilm was formed at 37°C for the extended time of 168 hours the mean density increased for 10 of the 13 strains. However, the large variation in measurements of biofilm density in replicate wells, even with an individual strain in a single microtitre tray experiment suggests that caution is needed in drawing conclusions from a limited number of experiments.

Biofilm formed in the microtitre plate was also treated with disinfectants sodium hypochlorite, sodium hydroxide and benzalkonium chloride for up to 90 minutes. The disinfectants were not effective at eliminating all viable cells from the microtitre plate system. This was broadly consistent with finding in the CDC biofilm reactor.

5.1. Introduction

It was demonstrated in chapters 2 and 3 that *Salmonella enterica* can form a biofilm on surfaces frequently associated with food processing environments. The results show that given suitable conditions such as nutrients and ambient temperature all 12 *S. enterica* strains examined form biofilm and that the biofilm density is related to the substratum it develops on. Overall, the results showed no major difference between the *S*. Agona strain related to the outbreak (SAGOXB.0066) and other strains of *S*. Agona. It was of interest to investigate biofilm density of these strains using alternative, widely used methods such as the microtitre plate based system.

5.1.1. Colony morphology of biofilm forming strains

As discussed previously in chapter 1, increased concentration of components of the extracellular matrix including curli and cellulose have been widely linked to increased density of biofilm formation [28, 77, 87, 94, 167]. The extracellular polymeric matrix components can be indentified through the use of congo red (CR) agar. This method involves growing the test organisms on an agar plate with a low salt concentration (Luria-Bertani Agar without salt) and with the addition of 2 indicator dyes, (1) congo red which binds to the amyloid protein fibres of the curli [222] and (2) coomassie brilliant blue dye which is a non specific protein binding dye that binds to curli and cellulose binding proteins [223]. The mixture of dyes facilitates the detection of both cellulose and curli fimbriae. The morphotypes displayed on CR agar are summarised in Table 5.1.

Binding of congo red and coomassie blue to the amyloid protein fibres of the cell (curli) and coomassie blue to cellulose leads to a red, dry and rough colony (rdar) morphology on the CR agar plate [77, 167, 224]. The brown

dry and rough (bdar) morphology occurs through congo red and coomassie blue binding to curli only (no cellulose binding protein). The pink dry and rough (pdar) morphotype is indicative of the coomassie blue binding to cellulose binding protein in the absence of curli. The finding binding of that coomassie blue only results in a pink colour may be a reflection of both congo red and coomassie blue contained in the one agar plate. The smooth and white (saw) morphotype is indicative of no binding of either the congo red or coomassie dyes [4]. Malcova and colleagues recently described the ability of *Salmonella* strains deficient of curli and cellulose to form a biofilm, due to over-production of a capsular polysaccharide resulting in a smooth, brown and mucoid colony morphotype (sbam) on CR agar [86].

Table 5.1: Colony Morpholgy Using Congo Red Agar

Morphology Name	Colony Morphology	Curli	Cellulose	Biofilm Former	Reference
rdar	Red, dry and rough colony morphology	+	+	Dense biofilm former	[77]
bdar	Brown, dry and rough colony morphology	+	-	Less dense biofilm former	[77]
pdar	pink, dry and rough colony morphology	-	+	Less dense biofilm former	[77]
sbam	smooth, brown and mucoid morphology	- capsular polysaccharide	-	Less dense biofilm former	[86]
saw	smooth and white morphology	-	-	Unable to form a dense biofilm	[77]

5.1.2. Previous research using the colony morphology assessment

Römiling and colleagues demonstrated that knocking out the gene encoding for curli expression (curli subunit genes *CsgA*) resulted in an altered colony morphology of *S*. Typhimurium on CR agar. The colony morphology changed from rdar to pdar based on this knockout process. This change was associated with less dense biofilm formation by strain [77, 167]. In this case, biofilm was examined through the use of a pellicle test (biofilm formed on air-liquid interface) cells on glass [77] and by a electron microscopic examination of stained biofilm [77]. Additionally, Römiling *et al.* demonstrated that mutation of the AgfD gene (involved in encoding for cellulose production) also altered the colony morphology resulting in a brown, dry and rough colony morphology (bdar) [167, 225]. The bdar strains also produced a more fragile biofilm than the wild type strain when examined using a pellicle formation model and assessing the force needed to disaggregate the pellicle [167, 225]. Moreover, as a result of this research, Römiling and colleagues demonstrated that the mutants lacking both genes in *S*. Typhimurium (LT2 mutants) were unable to form a biofilm and could be identified as smooth and white (saw) on CR agar [77, 167, 225] .

Malcova *et al.* recently reported that *S*. Typhimurium strains that do not appear to produce curli or cellulose based on morphology on CR agar, were capable of forming a dense biofilm using the microtitre plate based method [86]. The formation of capsular polysaccharide by the strains resulted in a smooth brown and mucoid colony morphology on congo red agar. Malcova *et al.* indicated that this phenomenon (production of a biofilm through the formation of a capsule without cellulose or curli) was not only associated with *S*. Typhimurium DT104, but was also found in 3 strains of *S*. Montevideo and 1 strain of *S*. Derby [86]. The presence of capsular polysaccharides that may be involved in biofilm formation in the

absence of curli and cellulose has also been reported elsewhere [82, 226]. This finding suggests that predicting biofilm formation based solely on identifying curli and cellulose producing strains by this method may not be reliable [82, 226].

Solomon *et al.* also used CR agar to investigate if the source of *S. enterica* strains was associated with variation in the level of curli and cellulose formation and the density of biofilm formed using the microtitre plate method [96]. The work included 15 clinical strains, 25 from multiple fruit/vegetable/seed sources (referred to as produce-related) and an additional 31 specifically from meat sources, incorporating 30 different serovars [96]. They reported that 80% of the produce-related strains and 100% of the clinical- and meat-related strains exhibited curli biosynthesis. Based on this, Solomon *et al.* suggested that morphological traits may be influenced by the source of the isolate [96]. In most instances, Solomon and colleagues found that the rdar or bdar strains were associated with more dense biofilm than the curli deficient (saw) strains when using Luria-Bertani (LB) media with additional glucose or a 1/20 dilution of Tryptic Soy Broth (TSB). Using the microtitre plate based method, there was no correlation between colony morphologies, the density of biofilm formed when using the recommended concentration of TSB. As a result, Solomon *et al.* concluded that biofilm density was more likely to be nutrient dependent than related to the morphotype on CR agar [96].

Castelijn *et al.* also found that *S*. Typhimurium formed a more dense biofilm in 1/20 TSB than in a full strength TSB when examined using a microtitre plate method [32]. Moreover, Castelijn *et al.* also found that these nutrient limited conditions induced curli and cellulose biosynthesis when examined microscopically [32]. This indicates that the expression of

curli and cellulose may be subject to environmental stress, such as low nutrient concentration.

5.1.3. Biofilm formation assessed using the microtitre plate method

The microtitre plate system is commonly used for the assessment of biofilm formation [32, 56, 82, 86, 96, 227]. This high throughput method is reported as rapid and reproducible for assessing the biofilm forming ability of a number of test organisms simultaneously. The multi-well structure allows multiple strains, and or multiple concentrations of nutrients or biocides to be assessed within one plate. However it is unclear to what extent results based on microtitre tray may be generalised to biofilm formed under other conditions [73, 102].

As described in chapter 1, the microtitre plate method is a static-flow biofilm model that allows biofilm development on the base of the multi-well microtitre plate made of plastic such as polystyrene. After incubation for a defined period of time (generally 24-48 hours), the inoculated media is extracted and the wells of the plate are stained with a bacterial staining agent such as crystal violet. Unbound crystal violet is removed through washing. The assumption is that the quantity of bound crystal violet is related to the density of biofilm formed on the microtitre plate. The bound stain is then resolubilized using an alcohol: acetone solution [56, 228] or glacial acetic acid solution [112, 165]. The optical density of the remaining crystal violet is then measured to determine the amount of biomass or biofilm in the wells of the plate.

Numerous authors have reported the use of *Salmonella* colony morphology on CR agar as a preliminary indication of strain biofilm forming capability before performing biofilm development studies using more conventional

studies such as the microtitre plate based system [82, 86, 96, 159]. As previously discussed Solomon *et al.* [96] and Castelijn *et al.* both [159] used the microtitre plate method to correlate rdar morphology and biofilm density. Castelijn and colleagues also found there was no significant difference between the density of biofilm formed on stainless steel coupons or plastic surfaces (using the microtitre plate method) [159]. However, the author found there were intra- and inter-serovar differences in the density of biofilm formed on the surface particularly for *S*. Typhimurium and *S*. Infantis [159]. In addition, Castlelijn *et al.* also found that when biofilm was assessed using a Live/Dead® fluorescent stain that biofilm density peaked after 24 hours incubation and declined in viable cells over the remaining 14 days. However, measurement of bacterial attachment to the surface after 24 hours may only reflect planktonic cell attachment as opposed to formed biofilm. Previously published research in this area used incubation times of ≥48 hours [56, 96, 111, 227].

Similar to Castelijn *et al.* [159], Díez-García *et al.* used a short incubation time of 24 hours to investigate biofilm density of 69 *S. enterica* strains using a microtitre plate based system and confocal microscopy [165]. Díez-García *et al.* reported that biofilm formation was influenced by the *Salmonella* serotypes, with the strains of *S.* Agona (*n*=3) and *S*. Typhi (*n*=1) forming a more dense biofilm than most other serovar groups including *S.* Enteritidis (*n*=36) and S. Typhimurium (*n*=3).

Bridier *et al.* also used a microtitre plate method to quantify biofilm formation for ten strains of *S. enterica* and 5 other bacterial species [178]. The strains of *S. enterica* included *S*. Agona (*n*=1), *S*. Enteritidis (*n*=1), and *S*. Typhimurium (*n*=2). Bridier and colleagues combined the used of the microtitre plate method with the use of confocal microscopy to measure biofilm density and assess the biofilm structure for the sixty bacterial

strains. However the method used by Bridier *et al.* differs substantially from other methods described in this chapter. In order, to attach a dye (Syto9 – green fluorescent nucleic acid marker), the bacterial attachment was interrupted after 1 hour when the wells were rinsed with 150mM of sodium chloride to eliminate any non-adherent cells. This was followed by adding sterile TSB and 5µl Syto9 to wells of the microtitre plate after which the plates were incubated for 24 hours at 30°C to allow biofilm development. This method is similar to that described by Peeters *et al.* who used a 4 hour initial incubation period before rinsing the wells of a microtitre plate when using a crystal violet and Syto 9 to quantify biofilm formation of clinically associated organisms [229].

As previously described in chapter 3, Vestby *et al.* used the microtitre plate method and a pellicle formation assay to investigate the biofilm forming capability of 21 *S. enterica* strains including *S*. Agona, *S*. Montevideo, *S*. Senftenberg and *S*. Typhimurium [56]. They concluded that particular strains which persisted in the environment were able to form a more dense biofilm than the other strains examined. Moreover, the results indicated that using this method, *S*. Agona and *S*. Montevideo strains formed a more dense biofilm than *S*. Typhimurium and *S*. Senftenberg [56].

5.1.4. Assessing the efficacy of disinfectants using the microtitre plate method

Although there is a substantial body of literature investigating *Salmonella* biofilm formation using the microtitre plate method in most instances the research is switched to detachable surfaces once large scale screening is complete and the research moves into disinfectant assays [56, 113, 134, 159, 227, 230]. Disinfectant assays are frequently performed on detachable surfaces suitable for traditional methods of plate count enumeration as

discussed in chapter 4. The reasons for the switch most likely relates to the fact that crystal violet staining stains all cellular biomass contained in the wells of the plates and therefore does not discriminate between viable and non-viable cells [231].

Alternative methods of using fluorescent stains to detect live cells [231] or both viable and non viable cells have been reported in recent years. Pitts *et al.* calculated percentage reduction of viable cells in a biofilm through measuring the absorbance of a cell respiratory stain (5-cyano-2,3-ditolyl tetrazolium chloride or CTC) post treatment with chlorine [231]. The percentage reduction in active metabolism was then used to evaluate the efficacy of the disinfectant treatments (reduction calculated from untreated wells – treated wells). This method was compared to the microtitre plate method (percentage removal of biomass) and enumeration of viable cells using the plate count method. The results suggest that there was an overall trend for all methods to display less viable biofilm cells (*P. aeruginosa* and *S. epidermidis*) after exposure to increased concentrations of chlorine. The percentage reduction using CTC corresponded with the mean $\log_{10}$ reduction results achieved through use of the plate count method, while the crystal violet methods did not show a similar level of reduction [231]. As discussed in chapter 4, according to the CEN standards EN13697:2001, a $4\log_{10}$ reduction in viable count is necessary to demonstrate the efficacy of a disinfectant against a bacterial suspension attached to a surface [133]. Although the criteria that can be applied to assess disinfectant efficacy by percentage using LIVE/DEAD staining is not defined is not defined in the CEN standard EN13697:2001.

Vestby *et al.* also investigated the use of synthetic furanone to inhibit biofilm formation using a microtitre plate based system [113]. They examined the effect of pre-exposure to furanone followed by treatment

with a 0.05% concentration of sodium hypochlorite and 0.02% concentration of benzalkonium chloride after biofilm development. The effect of furanone was assessed by measuring the optical density of biofilm formed on a microtitre plate, while the effect of the disinfectants on the established biofilm was measured through the use of plate count method [113]. The results indicated that pre-exposure to furanone increased the efficacy of the disinfectant agents. There was a ~2 $\log_{10}$ difference in the number of viable cells recovered following treatment with furanone and sodium hypochlorite in comparison to the disinfectant alone. There was also a ~1 $\log_{10}$ difference in the number of cells recovered following treatment with furanone and benzalkonium chloride compared with the benzalkonium chloride alone [113]. However, even the combined treatment with furanone and sodium hypochlorite only reached a 4 $\log_{10}$ reduction ($\log_{10}$ density of biofilm recovered from the untreated surface– $\log_{10}$ density of biofilm recovered from the treated surface) for 1 of the two strains of *S*. Agona examined [113]. Moreover, it is important to note that none of the agents used by Vestby *et al.* completely eliminated all viable cells from the biofilm [113].

Against this background the objectives in this chapter are to firstly examine if dense biofilm forming potential can be predicted using congo red agar and if this method can be used as an appropriate screening method to identify dense biofilm forming strains. Secondly, to investigate the use of the microtitre plate method to examine biofilm density of *S. enterica* strains and the ability of the strains to withstand disinfectant treatment using a microtitre plate based method.

5.2. Methods

5.2.1. Colony morphology on congo red agar

I. The test organisms were streaked onto Luria Bertani (LB) agar and incubated at 37°C.

II. Congo red (CR) agar was made in batches of 15 plates on two consecutive days. The CR agar consisted of LB agar without sodium chloride ($LB^{w/o}$ NaCl) which consists of bacto-tryptone (10 g/L), yeast extract (5 g/L) and agar (15 g/L). The agar was sterilized by autoclave before the addition of the dye agents:

  - Congo Red (40 µg/ml) and coomassie brilliant blue R (20 µg/ml).

III. The CR plates were streaked with colonies from the nutrient plate and incubated for 48 hours.

IV. The CR plates were examined and categorised as rdar, bdar, pdar, sbam or saw accordingly (see table 5.1).

V. The experiments were performed twice on all 13 strains.

VI. The *S*. Typhimurium strain LT2 was used as a positive control for the rdar morphology. As stains previously characterised as bdar, pdar and saw morphotypes were not available the colony morphology produced by each strain was compared to the published images (of colony morphology) provided by Römling *et al.* [77, 167] and Malcova *et al.* [86].

5.2.2. Microtitre plate method

I. The test organism was streaked onto LB agar and XLD Agar and incubated overnight at 37°C.

II. A colony from the plate LB was picked off the plate and diluted into 10ml LB broth without salt (LB $^{w/o}$NaCl).

III. The LB broth $^{w/o}$NaCl consisted of bacto-tryptone 10g/L, yeast extract 5g/L.

IV. The overnight culture of *Salmonella* was diluted in LB broth without NaCl to an optical density of 0·2 using the handheld OD meter (Siemens-Microscan Turbidity Meter).

V. Thirty micro-litres of the bacterial suspension broth was transferred to each well in a 96-well polystyrene microtitre plate containing 100 µl LB $^{w/o}$NaCl.

VI. The plates were incubated at 22 ± 2°C (monitored room temperature) or at 37°C for 2 and 7 days.

VII. During biofilm development over 7 days, the media was aspirated from each well every 48 hours and replaced with fresh sterile media (130 µl) using a multi-well pipette with sterile tips.

VIII. After 7 days of incubation, the plates were emptied and gently washed once with sterile distilled water.

IX. The biofilm was stained by adding 130 µl of 1% crystal violet (stock concentration) and incubated for 30 min at room temperature.

X. The plates were gently washed three times with sterile distilled water to remove excess dye.

XI. One hundred and thirty micro-litres of an ethanol/acetone (70:30 v/v) solution was added to each well to resuspend the bound crystal violet dye.

XII. The plates were incubated for an additional 10 minutes at room temperature before OD_{590} was measured.

XIII. The difference in optical density was calculated by subtracting the optical density of the control wells from the optical density of the test wells.

XIV. Optical density (OD_{590}) was measured using the Dynex Technologies model-Best 2000 and the data was accumulated using Revelation Software version 5.19.

XV. The experiments were repeated on three different days.

XVI. The data was analysed using SPSS version 20 using the same statistical tests as described in chapter 1 (mean, standard deviation and comparisons of data using Mann-Whitney U test for non-parametric data).

XVII. The strains were examined in three wells in each experiment and each experiment was performed on 3 different days. Therefore the mean and standard deviation were calculated on 9 optical density measurements (observations).

5.2.3. Disinfectant testing

I. The test strains were streaked onto LB and XLD plates and incubated at 37°C for 24 hours as previously.

II. The three disinfectants as detailed in chapter 4 were used in this chapter sodium hypochlorite (500mg/L), sodium hydroxide (1 Mole –M), and benzalkonium chloride (0.02%).

III. The disinfectants were made using sterile H_20 and filter sterilized compounds, which were made on each day of experimentation.

IV. A bacterial suspension was made to an optical density of 0.2 in sterile saline using a hand held OD reader.

V. The plates were filled in 2 batches of 12 with 130µl of the bacterial suspension (0.2OD).

VI. Six plates were incubated at room temperature (measured as ~22°C) or at 37°C for 48 and 168 hours.

VII. After 48 hours the 48 hour test plates were treated with disinfectant as described below.

VIII. After 48 hours incubation the spent media was extracted from each well in the 168 hour test plates and replaced with sterile LB $^{w/o}$ NaCl media. After the 168 hours had elapsed the plates were treated with disinfectants as described below.

IX. To ensure all cells in each well were treated with disinfectant the plates were treated with 200 µl of each disinfectant for the assigned contact times of 10, 45 and 90 minutes with the disinfectant agent.

X. After the time had elapsed the wells were drained of the disinfectant agent.

XI. The disinfectant treatment was directly followed by the addition of 200 µl of the corresponding neutralizing agent for 30 minutes.

XII. One hundred and thirty micro-litres of the sterile media was added to each well of the microtitre plate.

XIII. The plates were re-incubated at 22°C and 37°C accordingly.

XIV. After 24 hour incubation the media was extracted from each well and placed into the corresponding wells of a sterile microtitre plate to examine the turbidity the broth.

XV. Increased turbidity indicated that cells in biofilm had survived disinfectant exposure and were able to grow in the fresh broth.

XVI. No change in turbidity (in comparison to negative control wells) indicated that cells were no longer viable post treatment with disinfectant agents.

XVII. Three wells with un-inoculated LB broth were used as a negative control in all runs.

XVIII. The three disinfectant solutions were also used as a negative control in triplicate wells.

XIX. The experiments were performed twice on different days.

XX. The data analysis was analysed using SPSS version 20. Comparisons and significance tests were based on the Mann-Whitney U test for multiple comparisons.

5.3. Results

5.3.1. The colony morphology on Congo Red agar

Using the categorisation described previously (Table 5.1) all strains of *S. enterica* displayed similar colony morphology when grown on CR agar, exhibiting the red, dry and rough (rdar) morphology indicating curli and cellulose biosynthesis.

Figure 5.1: Images of colony morphology of on congo red agar

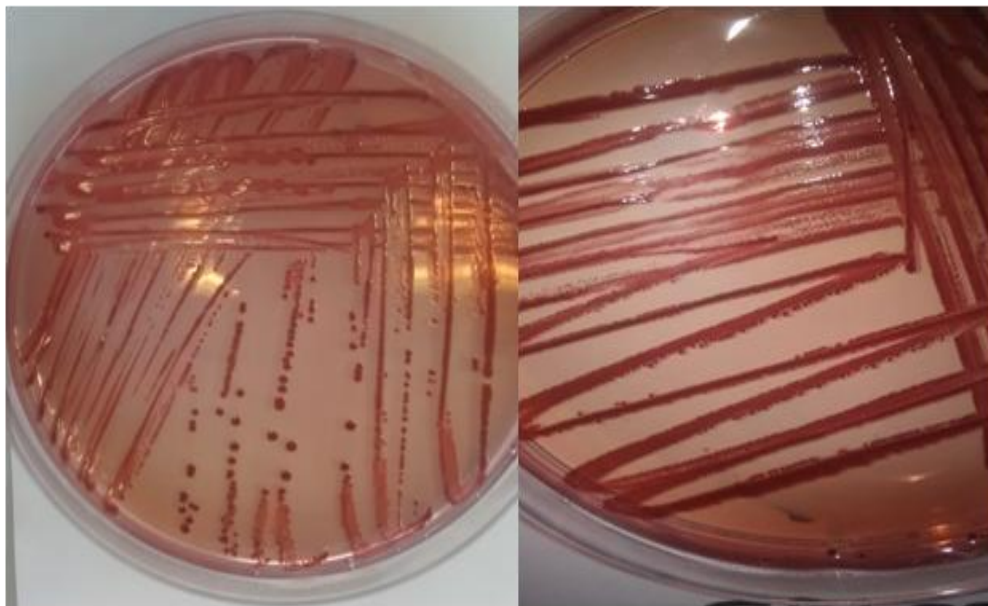


Figure 5.1 illustrates the red dry and rough (rdar) colony morphology displayed by *Salmonella enterica* subspecies *enterica* serovar Agona strain SL483 and *S*. Agona strain S09-0046.

5.3.2. Repeatability of measurement of Optical Density (OD)

In order to investigate difference in repeated measures (repeatability), the optical density of a single test plate with stained biofilm (as described in 5.2) was read twice consecutively at an optical density of 590 nanometres wavelengths (OD_{590}). As a representation of the variation in repeated measures of OD_{590}, the results of the repeated OD_{590} measurements for 6 *S.* Agona are displayed in Table 5.2. The results of the repeated OD_{590} measurements for all 12 *S. enterica* strains and the *P. aeruginosa* strain are displayed in Table 1 in the Appendix 2 of this thesis. As displayed in Table 5.2 in this chapter, the OD_{590} was read for each individual strain in three replicate wells. In most cases (12/18), the difference in OD_{590} on repeated measurement was less than 0.100 however it was in the range 0.1 to 0.3 for 5 wells and 1.369 for one well.

There was also a large difference in OD_{590} measurement between the three replicate wells (in same plate read) representing a single strain. For a number of strains there was at least a two-fold difference in measured OD_{590} for one well in comparison to the other 2 measurements of OD_{590} for the same strain (the full set of data for all 13 strains is available in the Appendices-Table 1). For the *S*. Agona S08-0601 strain, there was approximately a ten-fold difference in measured OD_{590} in one well in comparison to the other OD_{590} measurements of replicate wells (Table 5.2). There was also a large difference in OD_{590} measurements between the second well of the *S.* Agona strain S09-0371 and the other wells for the same strain, this was evident from both the first and the second read of optical density (Table 5.2). The variation in OD_{590} measurement from 3 replicate wells for each strain resulted in an elevated mean (higher than if these outliers were excluded), which is denoted by a large standard deviation of the mean for each strain which is further discussed in sections 5.3.3 and 5.3.4.

Table 5.2: The optical density of biofilm formed using the microtitre plate

	Strain	Well	1st OD_{590} Read	2nd OD_{590} Read	Difference (1st-2nd OD)
S. Agona	S08-0601	1	0.344	0.218	0.126
		2	1.641	0.272	1.369
		3	0.141	0.100	0.041
S. Agona	S09-0494	1	0.339	0.504	-0.165
		2	0.260	0.245	0.015
		3	0.130	0.113	0.017
S. Agona	S09-0046	1	0.157	0.179	-0.022
		2	0.091	0.107	-0.016
		3	0.057	0.074	-0.017
S. Agona	S09-0371	1	0.870	0.603	0.267
		2	1.119	1.112	0.007
		3	0.142	0.115	0.027
S. Agona	S09-0479	1	0.119	0.109	0.010
		2	0.074	0.072	0.002
		3	0.209	0.168	0.041
S. Agona	SL483	1	0.473	0.634	-0.161
		2	0.589	0.566	0.023
		3	0.819	0.571	0.248

Table 5.2 displays the optical density for *Salmonella enterica* subspecies *enterica* serovar Agona biofilm formed in 3 replicate wells for each of the 6 *S*. Agona strains examined. The optical density was measured at 590 nanometres wavelengths (OD_{590}). The measurement of OD_{590} was repeated on the same plate twice to examine the repeatability of measurement of OD_{590} values. Each strain was examined in triplicate wells in the microtitre plate. The values for the *S*. Agona strains are displayed in Table 5.2 however the values for all 12 *S. enterica* and the 1 *Pseudomonas aeruginosa* strain when the OD_{590} for each was measured twice in one plate are listed in Table 1 in Appendix 2.

Figure 5.2: Stained biofilm attached to surface (48 hours at 22°C)

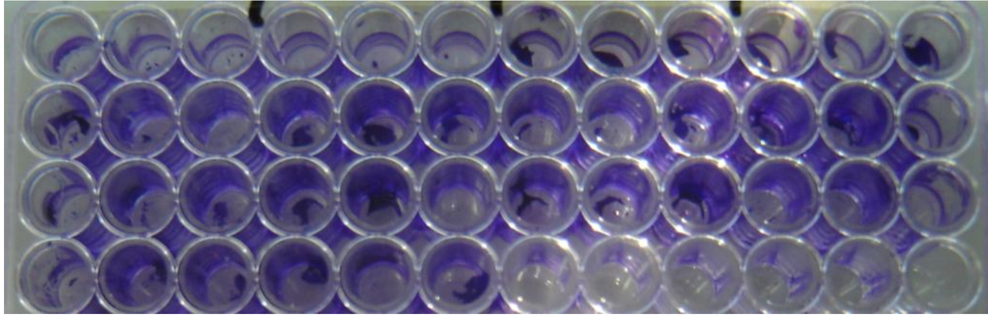


Figure 5.3: Stained biofilm attached to surface (48 hours at 37°C)

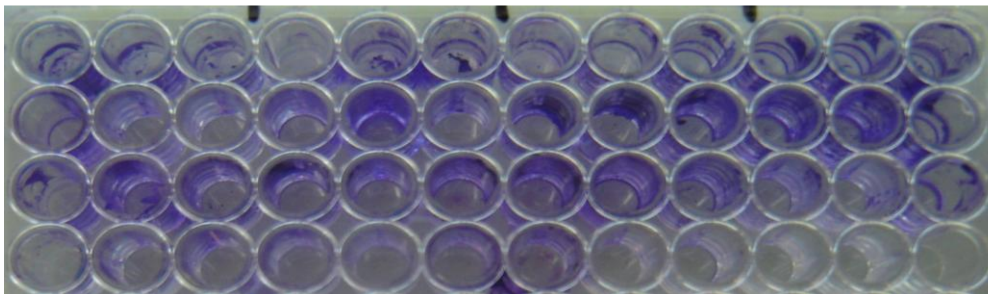
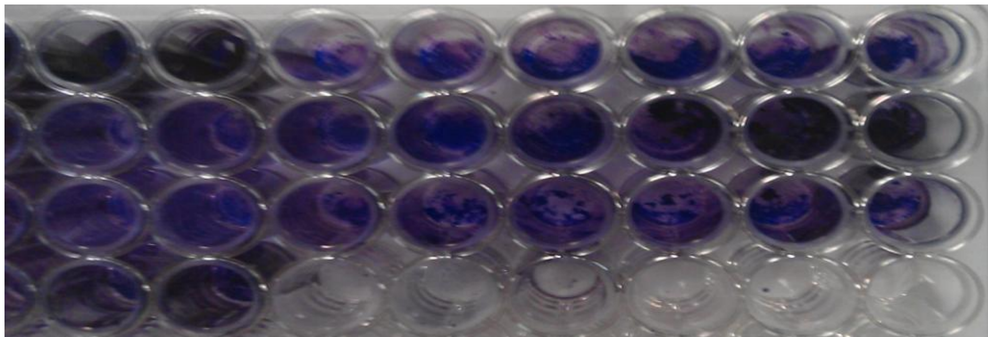


Figure 5.4: Stained biofilm attached to the surface (168 hours at 37°C)



Figures 5.2, **5.3** and **5.4** displays the crystal violet stain remained attached to the surface of the microtitre plate post resolublising the stained biomass with ethanol: acetone. The optical density was read with the ethanol: acetone solution in the wells after which the solution was extracted to examine the extent of crystal violet that failed to resuspend in the solution. The amount of crystal violet attached to the material in the microtitre plate is indicative of dye that failed to resuspend. The incomplete resuspension of the stained biofilm may have contributed to large variation in repeated measurements of optical density.

5.3.3. The density of biofilm formed over 48 hours

The density of biofilm formed by 13 strains was examined using the microtitre plate based method. The experiments were repeated on three occasions with 3 replicate wells used for each strain for each experiment. The results presented in this chapter (mean, standard deviation of mean (SD) and ranking of strains based on mean) are based on these 9 measurements of optical density. However, as displayed in Appendix 2-Table 2 and the SD's provided in tables 5.3-5.6 there were large variations in repeated measurements of optical density of stained biofilm measured for each strain. This variation is most likely related to uneven biofilm formation and incomplete resuspension of the crystal violet stain from the biofilm matrix. This incomplete resuspension is also displayed in Figures 5.1-5.3. The large variation in repeated measurement of optical density (denoted by SD) reduces confidence in the findings achieved when comparing mean optical density values. This is due to the fact that some of the mean values may appear to be elevated through the presence of a small number of outlying OD_{590} measurements. As a result, caution should be exercised in directly comparing the mean OD_{590} of the three serovars.

Table 5.3 presents the mean OD_{590} and standard deviation of the repeated OD_{590} for all 13 strains when biofilm was developed for 48 hours using the microtitre plate method. The mean and standard deviation are underlined for 3 strains (*P. aeruginosa* 27853, *S*. Agona S08-0601 and *S*. Typhimurium SL1344) due to the high OD_{590} for some replicate observations for these strains. The full set of 9 OD_{590} measurements for each strain is displayed in Appendix 2-Table 1.

There were a number of striking inconsistencies with respect to the two *S*. Agona outbreak strains S08-0601 and S09-0494 strains. When the biofilm

was formed at room temperature, the results suggested that the outbreak strains of *S*. Agona S08-0601 formed a more dense biofilm than other strains of *S*. Agona, *S*. Typhimurium and *S*. Enteritidis studied. However there appears to be relatively little difference between the mean OD_{590} of the stained biofilm when the strains were incubated at 37°C. When the full set of OD_{590} measurements were examined (Appendix 2-Table 2 and Table 3), the difference in mean was largely due to the impact of a small number of outlying values on the mean OD_{590} for the *S*. Agona S08-0601 strain. If those outliers were excluded there appeared to be only a marginal difference in the OD_{590} measurements of biofilm formed by the two outbreak strains. Similarly, there were only marginal differences between the *S*. Agona strains related to the outbreak and the other *S*. Agona strains (not associated with the outbreak).

However, when the biofilm was formed at 22°C in most instances, the *S*. Agona strains formed a more dense biofilm than the *S*. Typhimurium and *S*. Enteritidis strains. This observation is displayed the ranking of strains based on the highest mean OD_{590} (1) to the lowest OD_{590} (13). Based on this, 5 of the 6 *S*. Agona strains display the highest mean OD_{590} measurements. Although as previously discussed, the variation in repeated measures (as displayed in Appendix 2-Table 2 and Table 3) needs to be considered when making direct comparisons of mean OD_{590} of strains. However the trend for *S*. Agona to form a more dense biofilm than *S*. Enteritidis and *S*. Typhimurium is not consistent when the biofilm was formed at 37°C.

Similarly, there were differences between the strains within the *S*. Typhimurium serovar when incubated at 22°C, particularly for the *S*. Typhimurium strain SL1344, but again there were a small number of outliers in the 9 observations of SL1344 (Appendix 2- Table 2 and Table 3). The presence of outliers in the set of measurements of OD was also

indicated through a high standard deviation which is underlined in Table 5.3. If the outliers for SL1344 were excluded there was no apparent difference between this strain and the other *S*. Typhimurium strains. There were also no consistent differences between the strains when incubated at 37°C for 48 hours. There were no major differences in the density of biofilm formed by the two *S.* Enteritidis strains when incubated at either temperature. The S. Enteritidis strains had the 7th and 8th highest mean OD_{590} when incubated at 22°C and the 7th and 12th highest mean OD_{590} when incubated at 37°C for 48 hours.

The strains were to be grouped into the respective serovars as displayed in Table 5.4. There appeared to be a difference between the three serovars when incubated at 22°C for 48 hours, with *S*. Agona forming a more dense biofilm than the other strains. However, as previously stated the large variation in results (denoted by SD) reduces the confidence in this finding.

Table 5.3: The density of biofilm formed over 48 hours.

Strain Number	Organism	48 hours- 22°C				48 hours -37°C		
		N	Rank	Mean	SD	Rank	Mean	SD
27853	*P. aeruginosa*	9	**12**	0.184	0.07	**1**	0.540	0.40
S08-0601	*S.* Agona Outbreak	9	**1**	0.815	0.66	**3**	0.291	0.47
S09-0494	*S.* Agona Outbreak	9	**11***	0.204	0.07	**4**	0.227	0.10
S09-0046	*S.* Agona variant	9	**5**	0.386	0.35	**11**	0.104	0.06
S09-0371	*S.* Agona	9	**3**	0.450	0.37	**13**	0.091	0.04
S09-0479	*S.* Agona	9	**6**	0.351	0.25	**5**	0.187	0.13
SL483	*S.* Agona	9	**4**	0.405	0.19	**8**	0.149	0.08
S09-0419	*S.* Typhimurium	9	**9**	0.245	0.28	**9**	0.144	0.09
S08-0408	*S.* Typhimurium	9	**10**	0.224	0.11	**10**	0.114	0.08
SL1344	*S.* Typhimurium	9	**2**	0.723	0.50	**2**	0.294	0.51
LT2	S. Typhimurium	9	**11***	0.204	0.11	**6**	0.183	0.08
S09-0717	*S.* Enteritidis	9	**7**	0.285	0.19	**7**	0.159	0.12
S09-0004	*S.* Enteritidis	9	**8**	0.260	0.29	**12**	0.103	0.06

Table 5.3 displays the mean optical density of biofilm formed on the surface of a microtitre plate following incubation at room temperature (22°C) and 37°C for 48 hours. The optical density was measured at 590 nanometres in wavelengths (OD_{590}). Strains include 1 *Pseudomonas aeruginosa*, 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *Salmonella* Typhimurium and 2 *Salmonella* Enteritidis strains. Table 5.3 also displays the mean OD_{590} and standard deviation of the mean. The most dense biofilm former (based on mean) is ranked as number 1 followed the next strain to form a dense biofilm *et cetera*. An asterisk (*) denotes a shared ranking position. The mean OD_{590} and standard deviation are underlined for 3 strains due to large OD_{590} measurements for some replicate observations for these strains. The full set of observations for all OD_{590} measurements for all 13 strains is provided in Appendix 2- Table 2 and Table 3.

Table 5.4: The difference in biofilm formed by *S. enterica* serovars after 48 hours incubation

Serovar	*N*	48 Hours 22°C			48 hours 37°C		
		Mean	SD	*P*	Mean	SD	*P*
***S*. Agona**	**54**	**0.435**	**0.39**		**0.175**	**0.21**	
S. Typhimurium	36	0.349	0.36	0.080	0.184	0.26	0.827
S. Enteritidis	18	0.273	0.24	0.041	0.131	0.10	0.207
***S*. Typhimurium**	**36**	**0.349**	**0.36**		**0.184**	**0.26**	
S. Enteritidis	18	0.273	0.24	0.714	0.131	0.10	0.212

Table 5.4 displays the in mean optical density of biofilm formed for each *Salmonella enterica* subspecies *enterica* serovars after 48 hours measured at 590 nanometres in wavelengths (OD_{590}). Strains include 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *Salmonella* Typhimurium and 2 *Salmonella* Enteritidis strains. The mean optical density is based on 9 observations for each strain (including any outliers), therefore the mean OD_{590} for the 6 *S*. Agona is based on 54 observations *et cetera*. The standard deviation (SD) indicates that for most strains there is a large variation of measures of OD_{590} for the strains in each serovar group (large SD's are underlined). Significance of any difference between the OD_{590} readings was assessed using the Mann-Whitney U test (denoted through the use of *P* value). The mean OD_{590} of the *S*. Agona strains (highlighted in bold font) was compared to the mean OD_{590} of the *S*. Typhimurium strains and the mean OD_{590} of the *S*. Enteritidis strains. The mean OD_{590} of the *S*. Typhimurium strains (highlighted in bold font) was also compared to the mean OD_{590} of the *S*. Enteritidis strains.

5.3.4. The density of biofilm formed over 168 hours using the microtitre plate system

When *S. enterica* biofilm was developed over an extended period of time (168 hours at 22°C), a similar pattern emerged in that the OD_{590} readings for replicate wells of the same strain indicated that there was a large variation in repeated measurements. As displayed in Table 5.5, the mean OD_{590} and the standard deviation is underlined for 5 *S*. Agona strains due to a small number of high OD_{590} values for some observations for these strains. The mean OD_{590} of stained biofilm would be smaller if these outliers were excluded. The full set of observations for all 13 strains is provided in Appendix 2- Table 4.

When the mean density of biofilm formed by the strains was examined after 168 hours incubation there was evidence to suggest that some strains of *S*. Agona may form a more dense biofilm but overall this pattern was not consistent for all strains after incubation at 22°C. This trend was also not consistent for some strains when incubated at 37°C for the 168 hour duration (Table 5.6) most likely due to high and low outliers in some of the repeated reading of the OD_{590} of *S*. Agona biofilm. The full set of observations are provided in Appendix 2 – Table 5.

If the strains were to be grouped into the respective serovars as displayed in Table 5.6, the *S*. Agona serovar has the largest mean OD_{590} for all 3 serovars. As previously discussed, because of the variation in OD_{590} readings within individual strains caution should be exercised when grouping the strains together to compare the three *S. enterica* serovars.

Table 5.5: The Density of biofilm formed over 168 hours

Strain Number	Organism		168 hours- 22°C			168 hours -37°C		
		N	**Rank**	**Mean**	**SD**	**Rank**	**Mean**	**SD**
27853	*P. aeruginosa*	9	**3**	0.329	0.08	**3**	0.788	0.55
S08-0601	*S.* Agona Outbreak	9	**10**	0.193	0.12	**4**	0.699	0.89
S09-0494	*S.* Agona Outbreak	9	**5**	0.302	0.36	**1**	1.200	1.32
S09-0046	*S.* Agona variant	9	**6**	0.280	0.25	**6**	0.334	0.79
S09-0371	*S.* Agona	9	**2**	0.360	0.70	**9**	0.234	0.21
S09-0479	*S.* Agona	9	**4**	0.308	0.28	**11**	0.146	0.11
SL483	*S.* Agona	9	**8**	0.233	0.17	**2**	1.162	1.34
S09-0419	*S.* Typhimurium	9	**1**	0.368	0.15	**10**	0.147	0.17
S08-0408	*S.* Typhimurium	9	**7**	0.251	0.17	**13**	0.095	0.06
SL1344	*S.* Typhimurium	9	**12**	0.173	0.08	**12**	0.099	0.06
LT2	S. Typhimurium	9	**13**	0.166	0.09	**5**	0.519	0.69
S09-0717	*S.* Enteritidis	9	**9**	0.199	0.13	**7**	0.294	0.38
S09-0004	*S.* Enteritidis	9	**11**	0.173	0.09	**8**	0.235	0.26

Table 5.5 displays the mean OD_{590} of biofilm formed on the surface of a microtitre plate following incubation at room temperature and 37°C for 168 hours. Strains include 1 *Pseudomonas aeruginosa*, 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *Salmonella* Typhimurium and 2 *Salmonella* Enteritidis strains. Table 5.5 also displays the mean and standard deviation of the mean which was calculated on 9 observations OD_{590} (3 plates x 3 replicate wells in each plate). The most dense biofilm former (based on mean) is ranked as number one followed the next strain to form a dense biofilm *et cetera*. The mean OD_{590} and standard deviation is underlined for 6 strains due to the large OD_{590} for some observations for these strains. The full set of observations for all OD_{590} measurements for all 13 strains is provided in Appendix 2- Table 4 and Table 5.

Table 5.6: The difference in biofilm formed by *S. enterica* serovars after 168 hours incubation

Strain	*N*	168 hours 22°C			168 hours 37°C		
		Mean	SD	*P*	Mean	SD	*P*
S. Agona	**9**	**0.280**	**0.36**		**0.629**	**0.97**	
S. Typhimurium	9	0.240	0.15	0.392	0.215	0.39	0.036
S. Enteritidis	9	0.186	0.11	0.511	0.264	0.32	0.735
S. Typhimurium	**9**	**0.240**	**0.15**		**0.215**	**0.39**	
S. Enteritidis	9	0.186	0.11	0.308	0.264	0.32	0.090

Table 5.6 displays the in mean optical density of biofilm formed for each *Salmonella enterica* subspecies *enterica* serovars after 168 hours measured at 590 nm in wavelengths (OD_{590}). Strains include 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *Salmonella* Typhimurium and 2 *Salmonella* Enteritidis strains. The mean optical density is based on 9 observations for each strain (including any outliers), therefore the mean OD_{590} for the 6 *S*. Agona is based on 54 observations *et cetera*. The standard deviation (SD) indicates that for most strains there is a large variation of measures of OD_{590} for the strains in each serovar group (large SD's are underlined). Significance of any difference between the OD_{590} readings was assessed using the Mann-Whitney U test (denoted through the use of *P* value). The mean OD_{590} of the *S*. Agona strains (highlighted in bold font) was compared to the mean OD_{590} of the *S*. Typhimurium strains and the mean OD_{590} of the *S*. Enteritidis strains. The mean OD_{590} of the *S*. Typhimurium strains (highlighted in bold font) was also compared to the mean OD_{590} of the *S*. Enteritidis strains.

5.3.5. The difference in biofilm density formed over 48 and 168 hours

Table 5.7 displays the difference between the mean OD_{590} of measured biofilm formed after 48 and 168 hours. The difference was calculated based on the mean OD_{590} (of 9 observations of the density of biofilm for each strain) as detailed in Table 5.3 and 5.5. The large variation in repeated observations was previously discussed (5.3.3 and 5.3.4).

As demonstrated in Table 5.7 in most instances, the density of biofilm formed at room temperature (22°C) did not increase over the extended period of time. In most cases the mean density was marginally less (difference in OD_{590} <0.100). However, for 3 of the 9 strains the mean density was significantly less dense after the extended period of time ($p<0.05$), this is most likely due to the outlying values (denoted by underlined figures in Table 5.7).

In contrast, ten of thirteen strains formed a more dense biofilm over the extended period of time when incubated at 37°C for the 168 hours. In most cases, the difference over time was greater than the OD_{590} after 48 hours. This was also evident when the microtitre plates were examined after the crystal violet strain was treated with ethanol: acetone to resuspend the crystal violet stain (Figures 5.2, Figure 5.3 and Figure 5.4). The Figures (5.2-5.4) illustrate the incomplete resuspension of the crystal violet dye particularly in the 168 hour biofilm incubated at 37°C. This suggests that the biofilm development was uneven on the surface and that the biofilm formed was more dense after the extended period of time at 37°C. However, in most comparisons of the extent of biofilm formed after 48 and 168 hours, the difference was not significant ($p>0.05$).

Table 5.7: The difference in mean density of biofilm cells after incubation for 48 and 168 hours

Strain Number	Organism	Hours (h)	22°C		37°C	
			Difference	*P* value	Difference	*P* value
27853	*P. aeruginosa*	168h-48h	0.145	.004	<u>0.248</u>	0.233
S08-0601	*S.* Agona Outbreak	168h-48h	<u>-0.622</u>	.003	<u>0.408</u>	0.659
S09-0494	*S.* Agona Outbreak	168h-48h	0.098	.965	<u>0.973</u>	0.233
S09-0046	*S.* Agona variant	168h-48h	-0.106	.757	<u>0.230</u>	0.200
S09-0371	*S.* Agona	168h-48h	<u>-0.090</u>	.047	0.143	0.310
S09-0479	*S.* Agona	168h-48h	-0.043	.566	-0.041	0.566
SL483	*S.* Agona	168h-48h	-0.172	.038	<u>1.013</u>	0.038
S09-0419	*S.* Typhimurium	168h-48h	0.123	.034	0.003	0.269
S08-0408	*S.* Typhimurium	168h-48h	0.027	.965	-0.019	0.353
SL1344	*S.* Typhimurium	168h-48h	<u>-0.550</u>	.003	<u>-0.195</u>	0.122
LT2	S. Typhimurium	168h-48h	-0.038	.627	0.336	0.825
S09-0717	*S.* Enteritidis	168h-48h	-0.086	.566	0.135	0.508
S09-0004	*S.* Enteritidis	168h-48h	-0.087	.757	0.132	0.171

Table 5.7 displays the difference in mean density of biofilm formed by each strain when incubated for 48 or 168 hours at 22°C or 37°C. Strains include 1 *Pseudomonas aeruginosa*, 6 *Salmonella enterica* subspecies *enterica* serovar Agona, 4 *Salmonella* Typhimurium and 2 *Salmonella* Enteritidis strains. The difference between conditions (time) was calculated by subtracting the mean density of each strain and calculating the significance of any difference between the OD_{590} readings using the Mann-Whitney U test. However due to the variation in repeated measures of OD_{590} for each strain (based mean based on 9 observations for each strain) caution should be exercised when making direct comparisons. The strains with large variation in repeated measures of OD_{590} of biofilm are underlined. The full set of results (of repeated measures of OD_{590} nm) is provided in Table 2 and Table 3 of Appendix 2.

5.3.6. Examining the efficacy of disinfectant products against an established biofilm using the microtitre plate based system

Sodium hypochlorite (500mg/L), sodium hydroxide (1M) and benzalkonium chloride (0.02%) were not effective in eliminating all viable cells from the surface of the microtiter plate after an established biofilm was formed for 48 hour or 168 hour biofilm at 22°C and 37°C. The results presented in Table 5.8 summarises all conditions (2 temperatures and 2 durations). Despite the use of contact times of 10-, 45- and 90-minutes with the disinfectants, the turbidity of the sterile broth increased when incubated for an additional 24 hours (both at 22°C and 37°C), indicating that the cells associated with the biofilm remained viable and were capable of withstanding disinfectant treatment.

Table 5.8: Summary of assessment of growth in microtitre plate after disinfectant treatment

	Strain No	Sodium Hydroxide 1 (M) Mole per Litre			Sodium Hypochlorite 500 (mg/L) Milligrams per Litre			Benzalkonium Chloride 0.02% (w/v %) Percentage weight per volume		
		10 min	45 min	90 min	10 min	45 min	90 min	10 min	45 min	90 min
27853	*P. aeruginosa*	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S08-0601	*S.* Agona Outbreak	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0494	*S.* Agona Outbreak	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0046	*S.* Agona variant	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0371	*S.* Agona	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0479	*S.* Agona	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
SL483	*S.* Agona	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0419	*S.* Typhimurium	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S08-0408	*S.* Typhimurium	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
SL1344	*S.* Typhimurium	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
LT2	S. Typhimurium	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0717	*S.* Enteritidis	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
S09-0004	*S.* Enteritidis	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth

After contact with disinfectant and neutralizing agents, sterile broth was refilled into each well followed by incubation of the plates at 22°C and 37°C for an additional 24 hours. The broth was then placed into new microtitre plate to assess a change turbidity of the broth. An increase in turbidity indicated that cells remained viable after contact with the disinfectant agents. As displayed in Table 5.8 cells remained viable after treatment with all disinfectants. This was true for treatment of the 48 hour and 168 hour biofilm formed at both 22° and 37°C.

5.4. Discussion

The results from assessment of biofilm related properties with the use congo red agar demonstrated that all 12 *S. enterica* strains displayed a red, dry and rough colony morphology. The *P. aeruginosa* strain 27853 displayed a metallic shine on the surface of the colonies on the CR agar plate which was different than the *S. enterica* strains. This morphology was similar to the rdar morphology displayed by Udea *et al.* who also characterised the colony morphology of *P. aeruginosa* strains [232]. Which suggests the slight difference in colony morphology is most likely species/genus related.

The presence of the rdar morphology suggests that the strains synthesized both curli and cellulose [167]. It has been suggested that these components may support dense biofilm formation in comparison to strains lacking curli or cellulose [28, 77, 87, 167]. However, as all the isolates displaying the rdar morphology, it was not possible to demonstrate an association between the rdar morphology and increased biofilm density. The indication that all strains studied form curli and cellulose correlates with the finding that all strains were able to form a biofilm using the microtitre plate method and the CBR (chapters 2-4). Previous authors have suggested that dense biofilm formers can be successfully identified through examining colony morphology on CR agar [28, 77, 82, 87, 167, 226]. However, other authors have questioned the discriminatory power of this method suggesting that *S. enterica* biofilm formation may be more related to biofilm attachment substrata [67] or the nutrient content provided to the biofilm [32, 96].

There are also significant limitations to the use of congo red agar to identify biofilm components. Colony morphology is subject to reader

interpretation therefore in order to confirm the morphological traits, previously characterised strains that display the relevant morphotypes should be used as positive and negative controls. Positive and negative controls generally involved the use of well characterised strains verified to contain production of curli and cellulose such as *S*. Typhimurium 14028-1s (LT2) which displays the rdar morphotype and gene knock-out strains that display other morphotypes [77]. The *S*. Typhimurium LT2 strain was used as a rdar positive control in this research however strains characterised as pdar, bdar and sbam were not available. Although the images of colony morphologies published elsewhere [77, 86] were used as a reference, the lack of a negative control is a significant limitation to the morphology characterisation work described in this chapter. However characterising colony morphology without the use of controls has also been described elsewhere [86, 95].

The results of the microtitre plate method experiments suggested that particular strains of *S.* Agona may form a more dense biofilm under certain conditions. As displayed in Table 5.4 the *S*. Agona strains had the highest mean OD_{590} measurements 22°C for 2 days based on ranking the *S*. Agona strains had the 3rd, 4th, 5th and the 6th largest mean OD_{590}. However, when the same strains were incubated at 37°C this trend was less apparent. Moreover, when the strains were categorised into their respective serovars, there was no evidence to suggest that any differences between the serovars was significant (Table 5.5). A similar pattern of some *S*. Agona strains generally forming a more dense biofilm than strains of *S*. Typhimurium and *S*. Enteritidis was observed when the biofilm was formed over 168 hours, although this pattern was less consistent.

However, as displayed through the large standard deviation of mean OD_{590} measurements, it was evident that there was large variation in repeated

measurement of biofilm formed in replicate wells for the same strains. On examining the full set of observations for each strain (all provided in Appendix 2) it was clear that for a number of strains there were a number of clear outlier OD_{590} measurements much greater than the other OD_{590} measurements for the same strains from replicate wells. These outliers give an elevated mean for those strains. The inconsistency in repeated measurements for a given strain is a major limitation of the method as applied here.

The outliers are most likely representative of the uneven biofilm formation and incomplete resuspension of crystal violet from the surface of the microtitre plate when the optical density was measured. This finding is also illustrated in Figures 5.2-5.4. An uneven biofilm matrix or clumping of biofilm formed in a microtitre plate system has also been demonstrated elsewhere using confocal microscopy by Bridier *et al.* [178]. The work presented by Bridier *et al.* examined biofilm formation of 60 bacterial strains following fluorescent staining of the biofilm and construction of 3 dimensional images based on stacked confocal micrographs. The research demonstrated the uneven biofilm formation by *S*. Agona, *S*. Enteritidis and *S*. Typhimurium strains on the surface of the microtitre plate. This uneven distribution of biofilm formation was also visualised for other organisms. Moreover Bridier *et al.* indicated that of the 10 *S. enterica* serovars, *S*. Agona biofilm formed a distinctive macro-colony structure, which may also contribute to the clumping appearance when examined. Bridier *et al.* also demonstrated that to a lesser extent all *S*. Enteritidis and *S*. Typhimurium strains also formed an uneven biofilm [178].

The large range of optical density readings for each individual strain may be a reason why previous authors have chosen to use the standard error (dividend of standard deviation) to display the differences in repeated

measures of *S. enterica* biofilm formation [56, 96]. However, the use of standard errors to demonstrate variance between repeated measures in the absence of standard deviations or confidence intervals is not recommended [233-234].

Previous published material in this area by Lianou *et al.* has also reported large variation in *S. enterica* biofilm density using the microtitre plate method stained with crystal violet [191]. Lianou *et al.* reported large variation on mean OD_{590} measurements and standard deviations of *S. enterica* serovars similar to that reported here. However, the author does not indicate if inter-run variation (repeatability) may have been responsible for some of this variation or if the variation was more related to differences between the strains in each categorised serovar. Moreover, the author stresses the need for inclusion of multiple strains of *S. enterica* serovars in any comparisons of serovars to ensure that the data is an accurate representation of the variation in biofilm density [191].

Taking into consideration the variation in repeated measures, there is still evidence to suggest *S*. Agona strains may form a more dense biofilm than *S*. Typhimurium and *S*. Enteritidis strains, particularly after 48 hours incubation at 22°C (section 5.3.3). Similarly, Vestby *et al.* also found *S.* Agona and *S*. Montevideo formed a more dense biofilm than other serovars (*S*. Typhimurium and *S*. Senftenberg) using the microtitre plate method [56]. Although as previously discussed, the authors used the median measurements standard error to report the variation in the 3 repeated measures for each strain examined [56]. As a result, it is difficult to compare the results presented in this chapter to what was reported by Vestby *et al.* using the same method. However, the standard error from this work (data not shown) is similar to that reported by Vestby *et al.* which

indicates that the variation in repeated measures in most likely very similar.

As previously discussed Bridier *et al.* found that *S*. Agona formed a more dense biofilm than other *Salmonella* serovars, however the findings were based on a limited number of strains and the use of confocal microscopy as previously described [178]. As a result, it is difficult to make any direct comparisons of the findings presented by Bridier *et al.* to the results presented in this chapter. Díez-García *et al.* also found that *S*. Agona produced significantly more biofilm than any other strain ($p<0.001$) using the microtitre based system, with all 3 *S*. Agona strains being classified as strong biofilm producers [165]. The standard deviation for each strain was much lower than that reported in this chapter. This may reflect the variation in the method of measuring optical density [165]. Díez-García *et al.* incubated the plates for a shorter period of time (24 hours at 37°C) and fixed the plates with methanol (to ensure the biofilm was not removed with washing) before staining with crystal violet solution [165]. Moreover, the author also used a glacial acetic acid solution to resolubilise the crystal violet before reading the optical density [165]. The glacial acetic acid have resulted in a more completed and therefore a more even colour density in the wells which may have resulted in a lower variation in repeated optical density measurements [165]. This method has also been used elsewhere [111-112, 135]. If time permitted the use of glacial acetic acid to resuspend the crystal violet may have been explored.

Interestingly, when the biofilm was established at 22°C, only 4 of the 13 strains produced a more dense biofilm after 168 hours than after 48 hours. Previous authors have also suggested that the use of microtitre plate systems may have limitations due to a limited surface area indicating that the bacteria may reach a saturation level where the volume of attached

cells peaks [165, 191]. Diez-Garcia *et al.* suggested that although the number of planktonic cells may increase over time, the number of cells attached may not increase accordingly and be subject to the surface area provided [165]. However when the strains were incubated at 37°C biofilm produced by ten strains increased in density from 48 hours and 168 hours (although in most cases the increase was not significant $p>0.05$). The changes in density of biofilm over time, may suggest that the increased temperature may support bacterial biofilm formation over the extended period of time. Similarly Vestby *et al.* found that incubation of 9 strains of *S. enterica* over 2-, 3- and 4- days at room temperature 20±1°C in a microtitre plate based system only resulted in an increase in the density of biofilm formed only for *S*. Senftenberg strains, but no increase in the density of biofilm formed by the *S*. Agona and *S*. Typhimurium strains [56]. The results of this chapter contradict what was reported by Wong *et al.* using a MBEC method [149]. As previously discussed in chapter 4, Wong *et al.* also indicated that *Salmonella* biofilm density did not increase over an extended period of time (7 days) at 37°C [149]. The difference in biofilm development and measurement methods may have been responsible for the difference in findings.

It is also necessary to note that the amount of crystal violet dye bound is dependent on the quantity and composition of the extracellular polymeric substances (EPS) present in the biofilm. Therefore, it is possible that the change in optical density measurements of biofilm attached after 48 and 168 hours may be a reflection on this change in EPS and not relating exclusively to a change in the number of viable cells in the biofilm. This may impact on correlation of biofilm density assessed by viable counts.

The results of this chapter indicated that neither sodium hydroxide (1M) sodium hypochlorite (500mg/L) nor benzalkonium chloride (0.02%) were

effective in eliminating all viable cells when in contact with the 48 hour or 168 hour biofilm. In all instances the turbidity of the broth increased when incubated for an additional 24 hours (both at room temperature and 37°C) after treatment of up to 90 minutes contact time. This result is surprising consider sodium hydroxide was effective at eliminating all viable cells from the concrete surface as discussed in chapter 4. This point will also be discussed in further detail in chapter 6 where both methods are compared in greater detail. A summary of disinfectant work previously performed on established biofilm was also discussed in detail in chapter 4.

5.5. Limitations of the study

The microtitre plate method has particular advantages such as it is more suited to deal with large scale screening of isolates for biofilm capabilities than other methods such as the CBR. It is also less time consuming and more cost effective. Despite the notable advantages of high-throughput analysis, there are certain limitations as briefly summarised here as follows.

The range in repeated measure of OD_{590} of stained biofilm for particular strains was notably high. However, it would appear that other authors have made comparisons of data with a similar variation in repeated measures, with the use of standard error to indicate the amount of variation [56, 95].

Due to time constraints improving the repeatability through optimizing the biofilm formation and measurement method was not examined more closely. It was also not possible to read the optical density in a second plate reader to assess if this extent of variation represented a problem with performance of the plate reader used or an inherent issue with the method.

Previous authors have also used heat [159] or chemical fixation [165] in order to increase reproducibility of reading optical density of biofilm formed. However, this may result in an over estimation in the volume of biofilm cells to the surface as it is likely that planktonic cells may also be attached to the surface. It may have been of interest to investigate if the use of acetic acid to resuspend the dye as this may have been more effective than ethanol: acetone solution [111, 165].

5.6. Summary

S. enterica can form a biofilm using the microtitre plate method. Despite the limitations of reproducibility using the microtitre plate method there was some evidence to suggest that the *S*. Agona strains may form a more dense biofilm that strains of *S*. Typhimurium and *S*. Enteritidis, particularly at 22°C after 48 hours incubation. There is also some suggestion that in most cases the *S. enterica* strains examined over 168 hours did not form a more dense biofilm than the strain formed in 48 hours when incubated at room temperature (22°C) but did at 37°C. Therefore an increase in temperature may result in the density of biofilm formed on surfaces increasing.

Furthermore the results presented in this chapter indicate that *S. enterica* cells contained within a biofilm can remain viable after contact with three disinfectant agents commonly used in industry, despite the use of extensive contact times of up to 90 minutes. This may have seriously implication on both domestic and food processing facilities if a biofilm develops on the surfaces in contact with food.

Chapter 6

Discussion of *S. enterica* biofilm formation

6. Discussion

The European Food Safety Authority recently reported that *Salmonella* was the most frequently recognised agent involved in food-borne outbreaks in the EU [14]. A large proportion of Salmonellosis arises from consumption of contaminated eggs or meat products [5]. Barker and colleagues previously reported that work surfaces can become heavily colonized after processing with contaminated food [29]. Numerous food-borne outbreaks of *Salmonella* have been linked to contaminated processing areas including a processing line [33-34], utensils [35] and in food storage containers [36]. *Salmonella* attachment and survival in food processing environments may be assisted by the ability to form a biofilm.

As a result, there are a large number of studies investigating *Salmonella* biofilm formation on contact surfaces such as glass [56, 84, 102, 106, 134, 189], steel [147-148, 188, 207, 220] or using more high-throughput methods such as the microtitre plate based method [56, 86, 96, 111-112, 159, 165, 178, 191]. However due to number of studies performed using different laboratory models, various *Salmonella* strains and altered methodologies, there are substantial disparities in the results reported. Moreover, it is also difficult to assess the extent of repeatability of some experimental work on which conclusions are based.

Furthermore, based on guidelines provided by Association of Analytical Communities International (AOAC) in order to validate qualitative and quantitative assessment of food microbiology methods when examining organisms such as *S. enterica*, numerous strains representing different serovars should be used [235]. Lianou *et al.* also recently argued that it was vital for the credibility of researchers to demonstrate their finding of *S. enterica* biofilm properties on multiple strains of the pathogen [191].

However, a number of authors have described *Salmonella* biofilm formation with a limited number of strains [67, 71], with a single serovar [73, 98, 102, 106, 138, 147, 187-188, 190] or using a single biofilm substrata [111-112, 159, 165, 191]. The work presented in this thesis makes a useful contribution to the knowledge of *Salmonella enterica* biofilm density on surfaces, as multiple strains within three serovars of interest *S*. Agona, *S*. Typhimurium and *S*. Enteritidis, were compared.

6.1. Replicating food processing environments

There are inevitable difficulties in applying findings from research in controlled laboratory conditions to food processing environments although it is important that experimental design takes account of real world environments, in so far as possible. For example, the use of multiple surfaces may allow a more comprehensive understanding of biofilm density on surfaces frequently associated with food processing environments. The conditions used during biofilm studies such as the reactor model chosen, temperature, nutrient flow and concentration can influence the density of biofilm formed [32, 73, 102, 117, 188]. Therefore, the biofilm density was assessed for 12 *S. enterica* strains using the CBR and microtitre plate based models for 48 and 168 hours. The experiments were also repeated to investigate the repeatability and accuracy of the findings.

Previous research indicates that the properties of a *Salmonella* biofilm formed under a specific set of conditions may be surface specific [71, 95, 102]. Therefore, a practical approach was undertaken in this work to use a biofilm model that allowed inclusion of multiple surfaces commonly found in food processing environments to be assessed simultaneously within one

model. The combination of surfaces was chosen with a view to ensuring that the findings were relevant to food processing environments.

Chapter two presents an evaluation of the ability of *S. enterica* strains to form biofilm on glass, stainless steel, polycarbonate plastic, glazed tile and concrete after 48 hours and the density of the biofilm formed. Tile supported more dense biofilm formation than any of the other surfaces. This finding was consistent throughout the experiments as the number of cells recovered from the tile surface was higher than all other surfaces across all 13 strains tested and in each repeated test. Furthermore, when a biofilm was developed for an extended time of 168 hours, as discussed in chapter three, the association of increased biofilm density on tile was also evident.

Further research in this area may help to clarify the relationship between biofilm density and substrata. Previous research into the properties of biofilm substrata has suggested that surface roughness may play an important role in bacterial attachment [102, 164]. As described in chapter 2, when surface roughness was examined with atomic force microscopy (AFM) glass was the least rough followed by tile, steel, polycarbonate and concrete. It appeared that in general substrata with greater surface roughness were associated with greater mean log density of cells recovered from the surfaces. The exception was tile. Tile was more rough than glass but less rough than other surfaces however by AFM although it supported the most dense biofilm formation. This suggests there may be a general relationship between surface roughness and density of biofilm formed however other factors such as polarity may also contribute to initial bacterial attachment to the surfaces [100], which may contribute to biofilm formation. This or other properties may account for exceptional density of biofilm formed on tile.

It is of interest to examine if a difference in surface roughness of the same material may contribute to increased biofilm formation. Arnold *et al.* assessed the surface roughness through the use of AFM and reported the finish of steel did contributed to bacterial attachment and early biofilm development for poultry associated organisms [103]. However, previous research into the relationship between surface roughness and increasing bacterial attachment has been inconsistent. Barnes *et al.* found that the finish of a steel surface measured with fluorescent scanning microscopy did not have a significant impact on attachment of *Staphylococcus aureus, Pseudomonas fragi, Escherichia coli, Listeria monocytogenes, and Serratia marcescens* [164].

6.2. *S. enterica* biofilm density using the CBR

The results presented in chapter 2 indicate that all strains of *S. enterica* and the *P. aeruginosa* strains examined were able to form a biofilm on all 5 surfaces after 48 hours using the CBR. The results also suggest that overall, there appears to be limited difference between the *S.* Agona strains studied and that *S*. Agona SAGOXB.0066 strains did not result in a substantially more dense biofilm than other *S*. Agona strains. Moreover, in all instances where the difference reached statistical significance (p<0.05) the outbreak strain formed a less dense biofilm on the surface in comparison to the other *S*. Agona test strain.

S. Agona formed a more dense biofilm in comparison to *S*. Enteritidis after 48 hours development. The ability of *S*. Agona to form a more dense biofilm than strains of other *S. enterica* serovars has also been reported elsewhere using multiple methods, including a pellicle formation and the microtitre plate method [56, 165, 178]. It has been suggested that *S*. Enteritidis is a dense biofilm former elsewhere [67, 98]. The differences

between *S*. Agona and *S*. Typhimurium were more variable across all 5 surfaces with less indication of a consistent pattern. Based on the CBR results examining biofilm development after 48 hours, the evidence suggests that differences may be serovar specific, with large difference between *S*. Agona and *S*. Enteritidis and a less apparent trend between *S*. Agona and *S*. Typhimurium across all 5 surfaces tested. More cells were recovered from the S. Agona biofilm than *S*. Typhimurium biofilm on 3 of the 5 surfaces ($p<0.05$). On that basis, it is possible that if a less rigorous method such as a narrower range of substrata was chosen or less replicates of experiments were performed, one might have concluded that there was a significant difference between *S*. Agona and *S*. Typhimurium. Although, examining biofilm formation on all 5 surfaces provides a more robust interpretation of any differences in biofilm density.

6.3. Biofilm formation over an extended period of time

A subset of strains was used for examination of biofilm density using the 5 surface materials over an extended period of time of 168 hours. As discussed in chapter 3. The results indicate that the *S*. Agona strain S09-0494 formed a more dense biofilm that the *S*. Agona strain SL483 on all 5 surfaces. The *S*. Agona outbreak strain S09-0494 also formed a more dense biofilm than the two *S*. Typhimurium strains. However, the *S*. Agona reference strain SL483 produced a less dense biofilm in comparison to the two *S*. Typhimurium strains. This suggests that under certain conditions such as an extended duration of biofilm development, the density of biofilm formed may be strain specific as opposed to serovar specific which was previously reported in chapter 2. Therefore comparing serovars should include more than one strain in each serovar which has also been suggested elsewhere [191, 235]. Moreover, based on the findings of this thesis, it may be of interest to extend the duration for biofilm development as there was some evidence of differences between the strains of the same serovar (such as *S*. Agona strains SL483 and S09-0494) at 168 hours which

were similar at 48 hours. It is important to acknowledge however that given the number of independent CBR runs (3 at 48 hours and 2 at 168 hours) and the number of coupons counted, the results at 48 hours are more robust that those for 168 hours.

Another interesting finding from the 168 hour biofilm research was that the *S*. Enteritidis strain S09-0717 was a more dense biofilm former than the *S*. Typhimurium strains and the *S*. Agona strain on most surfaces when biofilm was developed over 168 hours. These findings are in contrast to what was reported from the studies performed using the CBR over 48 hours as reported in chapter 2. These findings indicate that the *S*. Enteritidis strain S09-0717 may be a slower biofilm former but given a longer period of time, the strain may form a more dense biofilm than most other strains examined. This interesting finding could have implications on the settings used for biofilm studies in the future. As most biofilm studies are based on short time scales, researchers in this area may be missing some important biofilm properties of the *S*. Enteritidis strains. Although caution needs to be exercised when making broad assumptions based on a small subset of strains as previously discussed. Ideally, to confirm these findings, more strains would need to be examined over an extended period of time.

Moreover, had the *S*. Enteritidis strain not been included in the experiments performed for 168 hours, it may have been assumed that the *S*. Agona outbreak related strain S09-0494 was the only strain that formed a significantly more dense biofilm in comparison to the other strains examined. For example, it may have easily been misinterpreted that the outbreak strain formed a more dense biofilm than the other strains examined - *S.* Agona SL483 and the two *S*. Typhimurium strains. This serves

to emphasise the importance of choosing multiple strains when designing experiments of this nature.

6.4. Biofilm formation using the microtitre plate method

It is important to note that any comparison made based on the mean OD_{590} data presented in chapter 5 are made with caution due to the substantial range of the 9 OD_{590} measurements for single strain. The large variation in repeated measures was much greater than that observed using the CBR (as indicated through standard deviations in each chapter). Variability may reflect uneven biofilm formation (and subsequent failure to resuspension in ethanol: acetone) by *S. enterica* in the microtitre plate method as suggested elsewhere [178]. However, it is also possible that some adaptation of the biofilm development method or evaluation of biofilm density may have resulted in greater repeatability however time constraints did not permit further optimisation of the method. Nevertheless, tentative suggestions of overall trends are made here based on those results. To describe the full extent of variation, the full set of OD_{590} measurements are provided in Appendix 2.

Overall, most of the *S*. Agona strains were more dense biofilm formers after 48 hours in comparison to most of the *S*. Typhimurium and *S*. Enteritidis strains. However, this trend was not consistent throughout, possibly due to the variation of OD_{590} of repeated measurements.

Interestingly, when the biofilm was established at 22°C only 4 of the 13 strains had a higher mean OD_{590} after 168 hours. There was no difference or only a marginal difference in the density of biofilm after extending the period of time to 168 hours (~0.1-0.2 mean OD_{590}). Two strains, *S.* Agona strains S08-0601 and *S.* Typhimurium SL1344 showed a larger difference in terms of mean OD_{590} over time. However the difference observed was

attributable to a small number of outlying high values for these strains which impacted significantly on the mean OD. If those outlying values were not considered the evidence of difference is much less. As discussed in chapter 5, the observation that *S. enterica* biofilm density does not increase over time using a microtitre plate method has also been reported elsewhere [56, 149, 165]. Previous authors have also suggested that the use of microtitre plate systems may have limitations due to a limited surface area indicating that bacteria may reach a saturation level where the volume of attached cells peaks over time depending on conditions [165, 191]. The small surface area of biofilm substratum may also not reflect conditions in food processing areas where the surface areas may be much larger and open to nutrient flow and contact with contaminants. However, this may be a limitation to all laboratory based biofilm development models as it may not be possible to replicate all biofilm development conditions found in food processing areas.

6.5. Overall comparison of biofilm development in CBR and microtitre plate

When the strains were assessed for biofilm development using the CBR in most instances the density of biofilm formed by *S*. Agona strains was greater than the biofilm formed by *S*. Enteritidis strains over 48 hours at 22°C. This broadly corresponds with what was reported using the microtitre plate based method for 48 hours (based on the mean density of the strains). However the results were less consistent using the microtitre plate method.

However, in many instances the results of the 168 hour biofilm formed using the CBR did not correlate with the findings of the microtitre plate method after 168 hours. The *S*. Enteritidis strain S09-0717 was a dense

biofilm former (in comparison to *S*. Agona SL483 and *S*. Typhimurium strains SL1344 and S09-0419) using the CBR, however this was not evident when using the microtitre plate system for the extended period of time. In general, the results of the microtitre plate method were broadly inconsistent.

Secondly, when using the CBR, the mean log density of cells recovered from the surfaces generally increased when the biofilm development time was extended from 48 to 168 hours. The microtitre results suggest in most instances the biofilm density did not increase for most strains after the extended period of time at 22°C. This result corresponds with what others have reported in this area [56, 149]. However the mean optical density of biofilm did increase over the extended period of time when the microtitre plate was assessed at 37°C. This suggests that given different conditions such as increased temperature or providing a larger surface area for biofilm development such as the CBR, biofilm density can increase over the extended period of time. This may have serious implications for the food industry if methods for reducing biofilm activity are based around the microtitre plate at room temperature it may underestimate the ability of strains to form more dense biofilm over an extended period of time or if the temperature in the environment increases.

6.6. The resistance of an established biofilm to disinfectant treatment

Disinfectant companies often market their products as active against *Salmonella* however these claims are often based on suspension test results only [134]. To investigate the efficacy of disinfectants in food processing and domestic environments, the methods applied should be as close to real life conditions as possible [130] . In light of this, the CBR was

chosen as an appropriate method for investigating the efficacy of disinfectants against a pre-established biofilm on concrete surfaces.

The results detailed in chapter 4 indicated that using the CBR, sodium hydroxide was effective at eradicating a 48 hour *Salmonella* biofilm for the two strains examined (*S*. Typhimurium SL1344 and *S*. Agona SL483). However, the same concentration of sodium hydroxide was not effective at eradicating all viable cells from a 168 hour biofilm of the same strains. Previous research has suggested that *Salmonella* biofilm density does not increase substantially over time and that the duration of development time does not affect treatment with disinfectant agents [56, 149]. Therefore, an important finding of this research was to demonstrate that age of biofilm may be important in studies investigating the efficacy of disinfectants against an established biofilm. When biofilm was formed using the CBR, there was a reduction in the number if viable cells directly after disinfection treatment with sodium hypochlorite and benzalkonium chloride. However, neither of the disinfectants was effective at eliminating all viable cells from the 48 hour or 168 hour biofilm. This highlights the need for careful consideration of content and concentration of disinfectants used for cleaning food processing environments that may be subject to contamination or presence of biofilm.

As described in chapter 5, when all 13 strains were assessed using a microtitre plate method, the results indicated that none of the 3 disinfectants were effective at eliminating the biofilm from the surface of the plate post 48- or 168 hour biofilm formation. This result was unexpected considering that sodium hydroxide was effective against the same bacterial strains when examined in the CBR system after 48 hours incubation. Although there were differences in the test procedures,

considering the same concentration of disinfectants and contact times were used in both it would be reasonable to expect a similar result.

The variation in methods may have contributed to differences in the apparent efficacy of the disinfectants against the biofilm formed. After the biofilm was treated with sodium hydroxide the biofilm was removed from the surface through sonication and cells were enumerated by viable counts using the plate count method. Therefore one hypothesis is that cells were viable on coupons after sodium hydroxide exposure but were more vulnerable to the effects of sonication used to detach biofilm from the coupon. However this would not explain why no viable cells were recovered from a 2nd set of coupons treated with sodium hydroxide that were re-incubated in broth post treatment (no sonication).

The difference in efficacy of sodium hydroxide may also be biofilm substratum related. The biofilm was formed on concrete using the CBR whereas the microtitre plate consists on polystyrene plastic wells. Polystyrene as a substratum was not studied in the CBR although polycarbonate plastic was involved in the CBR studies. The results presented in chapters 2 and 3 of this thesis indicated that the biofilm substratum can influence the density of biofilm formed. Previous authors have also suggested that difference in surface structure such as surface roughness may allow for resistance to disinfectants [102]. Korber *et al.* found the presence of crevices in glass resulted in increased bacterial survival compared with a smooth glass surface. It is also possible that the conditions for media flow and shear stress on biofilm formed in the CBR impacted in some way to enhance the porosity of biofilm allowing more rapid penetration of sodium hydroxide solution to all parts of the biofilm and therefore more efficient killing.

The reason why sodium hydroxide was effective against biofilm formed in the CBR but not against biofilm formed in microtitre plate remains

undetermined. However, the fact that the disinfectants were not effective at eliminating the biofilm using the microtitre plate method cannot be overlooked. Perhaps this unexpected result serves to emphasise the necessity to consider multiple experiments and conditions when examining the efficacy of disinfectants against a pre-established biofilm. The broad findings from both methods indicate that the three disinfectants are of limited use against an established biofilm. This also correlates with indications that eradication of the *S*. Agona SAGOXB.0066 associated with the outbreak from one area on the food campus using a range of treatments including sodium hydroxide, vaporised hydrogen peroxide and heat treatments was challenging (personal communication [58]).

6.7. Investigating repeatability of measuring biofilm density

6.7.1. Repeatability of results using the CBR

If the extent of variation that occurs with a single strain on a single substratum is not established, there is a risk of interpreting differences between strains or substrata as significant when it may only have occurred through random variation. Moreover, the extent of variation may also account for some apparent inconsistencies in the reported data on biofilm development. As a result, it is important to investigate the limits of repeatability in an experimental system. Repeatability refers to the ability to produce the same results during a repeated experiment within one laboratory [236].

The International Standards Organisation ISO (157251:1) defines repeatability as the standard deviation of results achieved through repeated experimentation under the same conditions [236]. The results of chapter 2 relating to the CBR indicate that the standard deviation varied with the strain and the surface tested. Based on the mean log density values, the range of SD values for all 13 strain was between 0.19-0.66 on

glass, 0.18-0.94 on steel, 0.17-0.70 on polycarbonate, 0.05-0.68 on concrete and 0.08-0.28 on tile (figures based on results presented in chapter 2, Tables 2.3-2.5). The SD varied between the 13 strains and there appeared to be no pattern of larger or smaller SD values for any particular strain or serovar.

As described in chapter 2, the previous research performed using the CBR [72, 117, 124] suggests that the repeatability, when measured using the SD, is similar to what has been achieved in this thesis. However, in most instances the research to date has examined a limited number of replicate surfaces or repeated experiments. It is also evident that the large number of strains tested in this thesis and the 5 surfaces examined simultaneously allow for the most comprehensive investigation to date of the density of *Salmonella* biofilm formation on multiple surfaces and of the variance of repeated measures using the CBR.

Joseph *et al.* investigated the biofilm density of 3 *S. enterica* serovars on concrete, plastic and steel but did not report the SD of the duplicated experiment [71]. Nevertheless the work performed by Joseph *et al.* is widely used as a reference to demonstrate biofilm formation on contact surfaces and resistance to sanitizers. The repeatability of measurements when examining the efficacy of the disinfectant against a pre-established biofilm in this study was similar also to that reported elsewhere [117, 134, 150].

Interestingly, the SD values were much lower when assessing log_{10} reduction of viable cells attached to concrete post treatment (details in chapter 4) than when assessing mean log_{10} density on 5 surfaces simultaneously, as described in chapter 2 and chapter 3. It is possible that a

reason for this may be due to only one surface (concrete) contained within the reactor, which may reduce variability due to mixing of surface associated properties contained in the enclosed reactor environment.

Alternatively a reason for variation may be due to a less robust methodology in determining the true extent of variation of mean log_{10} density. When mean log_{10} density was assessed, as described in chapter 2, the mean log_{10} density was based on 9 observations (counts performed once on each of 3 coupons x from 3 separate CBR runs) while the method described in chapter 3 was based on 6 observations (counts performed once on each of 3 coupons x from 2 separate runs). The results presented chapter 4 were based on 6 observations (counts performed 3 times on 1 coupon x from each of 2 runs). This change was made to manage the time available to complete the experiments however it also serves to illustrate the extent to which the full extent of variability within a single strain may be underestimated by limitations of methodology. In the latter experiments because of using fewer coupons the difference in repeated measures (represented by SD) was smaller. The use of a larger number of coupons will provide a more comprehensive indication of the ruggedness of the method and the extent of variation between repeated measures of the same strain (repeatability). Therefore multiple measurements are required to confirm apparent differences of biofilm density between strains examined and differences reported based on less robust methods may be questionable.

To summarize, the investigation of repeatability of the research performed on multiple contact surfaces using the CBR, measured by SD values, appears to correspond with what has been reported elsewhere. Furthermore the data presented here enhances the understanding of the density of *Salmonella* enterica biofilm formation through the use of

multiple strains and using a number of food contact surfaces simultaneously which help to mimic real life conditions. An unequivocal basis for conclusions regarding phenotypic differences between strains in respect to biofilm formation or biofilm density are an essential preliminary step to studies intended to elucidate molecular studies that underlie such differences. However this requirement is not always met in published studies. One factor contributing to this situation is the workload involved in the phenotypic work.

6.7.2. Ruggedness and Reproducibility

The term ruggedness signifies the degree of variability in results (mean log $_{10}$ density) after slight modification of the method such as temperature change or flow rate [72]. The research performed by Goeres *et al.* also indicated that there was slight variation in the mean $\log_{10}$ density of *P. aeruginosa* ATCC 700888 biofilm formed depended on the position on the arm within the reactor ranging from 0.08-0.18. The mean $\log_{10}$ density of *P. aeruginosa* biofilm was 5.63 ±0.17 CFU cm^2 using the standard protocol. The mean $\log_{10}$ density also increased when the batch mode increased by 2.4 hours (0.05 cfu/cm^2) and a 10% decrease in nutrient concentration resulted in a mean $\log_{10}$ reduction of 0.10 cfu/cm^2. Additionally a 2°C increase in temperature increased the mean $\log_{10}$ density by 0.04 cfu/cm^2 while increasing the rate of revolution of the baffle by 10 rpm increased the mean $\log_{10}$ density by 0.09 cfu/cm^{-2}. In total, 21 experiments were performed and the results were assessed on triplicate coupons taken at random from each run. Replicate experiments were undertaken to investigate the reproducibility under each alternative set of conditions. Overall, the research on optimizing conditions for the use of the CBR suggest that the method was reproducible and sufficiently rugged to withstand slight modification of the procedure [72, 236].

Moreover, based on the results presented in this research, the CBR appears to be a much more reproducible method for examining biofilm density that the use of the microtitre plate. However, if there was more time available to work on this project it may have been useful to examine if the extent of variation in repeated measures of biofilm formation using the microtitre plate could be improved by optimizing the formation and enumeration methods as previously discussed in chapter 5.

6.7.3. Heterogeneity of the surfaces.

Due to the process of manufacturing certain materials such as concrete the surface roughness may vary even between coupons of the same material. As described in the ISO standard 5725-2:1998(E), the use of sand granules in the manufacturing of concrete means there may be gradation in the size of particles between each sample which may result in slight variation in surface area . In addition, the process of producing bulk materials such as the steel, tile or polycarbonate may also be subject to slight variation between the finished surfaces. Of all five surfaces examined using SEM analysis, glass appeared to be the most homogenous surface. The glass surface also had the smallest standard deviation when surface roughness was assessed using AFM, as described in chapter 2. The surface heterogeneity may contribute to some of the variability observed. However depending on the nature of the experiment and the variability in results, surface heterogeneity may be an important consequence to consider in experimental design. As an example, if the surfaces vary in a laboratory model, it may be reasonable to assume that a similar degree of variability may be present in real life environments. As discussed in chapter 2, the extent of surface roughness may increase the density of biofilm formed on some of the surfaces. Therefore difference in surfaces may need careful consideration when choosing biofilm substrate for research of this nature.

6.8. Limitations of the Studies

6.8.1. Incomplete removal of biofilm cells from the surface

Scanning electron microscopy was used for all experiments performed using the CBR to assess completeness of biofilm removal from the surfaces by sonication. Based on the evaluation of all 13 strains investigated for biofilm formation over 48 hours, only one strain (*S*. Typhimurium LT2) was not fully removed using the conditions described in. This finding highlights the need to use SEM or other forms of microscopy to confirm removal of biofilm from a surface which is used for enumeration and comparative purposes as conclusions based on inconsistent removal are more difficult to interpret.

6.8.2. Incomplete removal of the mature biofilm

As discussed in chapter 3, sonication under the conditions described was used to maximise removal of biofilm from the surfaces post developed over 168 hours. The results of chapter 2 and 3 indicate that sonication removed more biofilm from the surface than swabbing the surface but that increasing the sonication power or duration was not as effective as it impacted on cell viability. As a result, incomplete removal decreases the validity of using a plate count method to determine the density of 168 hour biofilm attached to the surface. If viable counts are to be applied to assess density of such biofilm then development of acceptable methods to achieve full removal without impairment of cell viability are necessary. An alternative is to work on developing methods that achieve enumeration of viable cells without biofilm detachment such as the use fluorescent staining, which also has certain limitations as discussed in chapter 2.

Due to incomplete removal of the 168 hour biofilm, direct comparisons (biofilm $\log_{10}$ density levels after 48 and 168 hours) should be looked at with a degree of caution. Incomplete but variable removal may be

responsible for increased variation between experiments which further demonstrates the importance of confirming removal from the surface. Giaouris *et al.* also noted incomplete removal of a 168 hour *S.* Enteritidis biofilm attached to stainless steel [188]. However, as described in chapter 3, the used of conductance measurement in broth may also have certain limitations.

In the absence of direct visualisation to confirm biofilm removal from the surface, researchers should be aware that reporting a mean $\log_{10}$ density of biofilm or a reduction in density of viable cells after treatment, solely reflects the cells removed from the surface [183]. Previous authors have described the use of sonication to remove biofilm for enumeration purposes without providing evidence that the entire biofilm was removed from the surface [71-72, 114, 118, 122, 124].

6.8.3. Biofilm density variability using the microtitre plate based method

As described extensively in chapter 5, the SD of the OD_{590} readings suggests a substantial range in values of the repeated measurement. The SD of the mean using the microtitre plate method was substantially higher than the SD reported with the CBR, as detailed in chapters 2 and 3. This indicates that limitations of repeatability of the microtitre plate method, as applied here, are such as to limit the application of the method for quantitative purposes. Similar variation on repeated measurements of optical density has been reported elsewhere using the microtitre plate model described in this thesis [56, 96, 191]. A number of authors chose to use the standard error (dividend of standard deviation) rather than the standard deviation to display the differences in repeated measurements [56, 96]. However the use of standard errors to demonstrate variance between repeated experiments is not recommended [233-234]. It is also useful to note that the mean and SD values reported in this thesis were calculated on 3 replicates test wells of three independent experiments (n=9), while

previously published material has published on 3 replicate wells from one experiment or have not provided the figures to examine the extent of variation in tabular format (graphs only) [56, 178].

6.8.4. Reasons for variation using the microtitre plate bases system

As discussed in the limitations of the research performed in chapter 5, there may be a number of reasons for this variation in SD values. First, it was evident from repeated examination that the optical density reader may not provide consistent values even on repeated examination of the same microtitre plate (see Table 5.1 in chapter 5 and Table 1 in Appendix 2 for details). It is also possible that slight variation in the position held in the automated reader may result in a different area going under examination. Low speed shaking of the inoculated microtitre plates during incubation may alleviate some aggregation of biofilm cells, however the aggregation may also represent the heterogeneous formation of macro-colonies on the surface. Moreover, post staining with crystal violet, it was apparent that the crystal violet stain was not completely eluted from the biofilm uniformly across the entire surface of the plate. It is possible that the use of other reagents such as glacial acetic acid [111-112, 135, 165] instead of ethanol: acetone may have been more effective. Others have achieved greater repeatability using this method [111-112, 165]. Due to time constraints this method could not optimized for the research presented in chapter 5.

6.8.5. The limitations of using repeated measurements

Although the level of repeatability presented in this work, broadly corresponds to what has been reported elsewhere, there is still a need to exercise caution when interpreting results. Due to the small sample size (6-9 observation in most comparisons of individual strains), the findings may

not fully capture the range of run-to-run and coupon to coupon variation. Ideally, a larger number of replicates would be used to validate the accuracy and trueness of the observations as described in the ISO standards 5725-2:1998(E) [236]. However in most instances, a compromise between repeated measures of the same experiment and measures of different parameters (strains, conditions, duration of biofilm development) also needs to be reached. This thesis represents one of the most rigorous evaluations of repeatability of measurement of biofilm studied in the CBR and in other biofilm reactor models examining *Salmonella* biofilm density attached to surfaces.

6.8.6. The use of multiple comparisons in order to extrapolate any differences

P values of 0.05 are generally accepted as indicating that the observed degree of difference is unlikely to have occurred by chance alone. However, given that multiple comparisons were made (up to 40 comparisons in some tables), this criterion of significance should be applied with caution. The degree of caution necessary when performing multiple comparisons is because the probability of finding a *p* value of <0.05 by chance alone increases as a larger number of tests are performed. In order to overcome this bias, a reader must understand, that the suggestion of a statistically significant difference may not necessarily be indicative of a meaningful difference.

In order to overcome issues relating to multiple comparisons, the Bonferroni correction can be applied where the significance threshold would be divided by the number of comparisons made [67]. However, this may result in real difference being overlooked as discussed in chapter 2.

6.9. Main Findings Summary

Previous research examining *Salmonella* biofilm density has tended to focus on a limited number of strains of interest [67, 98], with a single serovar [67, 102, 106, 138, 148, 187-188] or using a single biofilm substrata [111-112, 165, 191]. However, it is difficult to form well founded conclusions if the strains are not compared to other strains and serovars under a variety of conditions and with consideration for repeatability of findings under a given set of conditions. It is of value to consider the surfaces commonly found in food processing settings. The work presented in this thesis enhances the knowledge of *Salmonella enterica* biofilm density on surfaces, as multiple strains within three serovars of interest *S*. Agona, *S*. Typhimurium and *S*. Enteritidis were compared with more thorough assessment of repeatability than is evident in most published research.

The main purpose of the current study was to determine if the *S*. Agona outbreak strains SAGOXB.0066 formed a more dense biofilm than other strains of *S*. Agona and/or other serovars of *Salmonella* commonly associated with foodborne outbreaks. The results show no convincing evidence to demonstrate that the outbreak strain or the variant strain (related to the outbreak) formed a more dense biofilm than other recent strains of *S*. Agona. This was demonstrated using the CBR and the microtitre plate method.

When the biofilm was developed over 48 hours the results suggest that *Salmonella* biofilm density may be related to serovars. The results indicate that in most cases *S*. Agona and S. Typhimurium may tend to form a more dense biofilm than *S*. Enteritidis. These findings were broadly consistent when examined using both the CBR and the microtitre plate method.

However, the extent of biofilm formed by the strains was also influenced by the method and conditions chosen.

Furthermore it was identified that other factors such as biofilm substrata and duration of biofilm development influence the density of biofilm formed. Biofilm substrata in particular had a major and consistent impact on the density of biofilm formed. More cells were consistently recovered from tile than concrete, glass, steel and polycarbonate. This indicates that surface roughness alone may not provide a clear indication on the ability of surfaces to support dense biofilm formation. Moreover, there is limited work examining *S. enterica* biofilm formation on tile, on the other hand, there is a great deal more research reporting biofilm density of glass [56, 84, 106, 134, 189], steel [148, 188, 207, 220] and plastic surfaces [56, 86, 96, 111-112, 159, 165, 178, 191]. The finding that *S. enterica* formed a more dense biofilm on tile that any of the other surfaces used in biofilm suggests the relationship between biofilm substratum and *S. enterica* biofilm density may be currently underestimated. This may have important implications for the food processing and handling industry suggesting that it may be necessary to consider the appropriateness of having materials such as tile in the food processing areas. It is also important to highlight that all materials were able to support biofilm formation using the CBR, this may also need consideration as many of these products are used in the food manufacturing industry.

Furthermore, the results also indicated that *Salmonella* biofilm density increased over time using the CBR. The research presented in chapter 4 indicated that the biofilm developed over an extended period of time was more resistant to disinfectant treatment. Previous research has suggested that *Salmonella* biofilm density does not increase substantially over time

and that the duration of development time does not affect treatment with disinfectant agents [134, 149].

The research presented in this thesis indicates that under the conditions of the microtitre plate method, *Salmonella* biofilm density does not increase for most strains with extended incubation [134, 149]. However, under the conditions examined using the CBR, in most cases the density of biofilm formed increased significantly. Particularly for the *S*. Agona outbreak strain S09-0494 and the *S*. Enteritidis strains S09-0717. Further studies into this area may have allowed a better understanding into the ability of certain strains to develop into a more dense biofilm over the extended duration of time. Further studies may have also aimed to examine if the use of the microtitre plate is an appropriate method for examining the number of viable cells or if it is more of a representation of stained biofilm matter (including dead cells and extracellular material) that may not be an accurate reflection of increased cell density over time. This may also provide evidence to indicate the usefulness of comparing the results achieved by the CBR and microtitre plate methods.

None of the three disinfectants were able to eradicate viable cells from a 168 hour biofilm using the CBR or the microtitre plate method. Studies on the efficacy of disinfectant agents intended for application in the control of biofilm should include assessment against an established biofilm, in particular a biofilm formed over an extended period of time under a number of different conditions. Development of a biofilm over an extended period of time on food contact surfaces may contribute to bacterial contamination of the surfaces, particularly if the disinfectants used do not eradicate all viable cells as demonstrated in this work.

6.10. Conclusions and recommendations

This work indicates no clear repeatable consistent differences between the *S*. Agona outbreak strain SAGOXB.0066 and other strains within *S*. Agona in terms of density of biofilm using the standard biofilm development methods. However, this work did demonstrate that given adequate access to growth area and nutrients all of the *Salmonella* strains were able to readily form a biofilm at room temperature on all surfaces tested. Furthermore, the strains of *Salmonella* examined were able to continue to proliferate over an extended period of time using the CBR method. It is plausible that any of the strains studied could lead to a large food-borne outbreak if not eliminated entirely through disinfection. It has also been demonstrated that it is difficult to eradicate all viable cells from a pre-established biofilm. This was also suggested from the findings of previous studies relating to *Salmonella* Agona outbreaks including the SAGOXB.0066 outbreak.

Therefore, it may be best to conclude that prevention of the introduction of *Salmonella* and the establishment of biofilm in a food processing environments is critical, as the biofilm may be difficult, if not impossible to eradicate with available methods once well established over time.

6.11. Further research

This research has highlighted many areas that would benefit from further investigation. It would have been of interest to investigate other areas surrounding this project such as the biofilm density of more *S. enterica* strains involved in food-borne outbreaks and sequenced strains. Additionally, it may have been of interest to investigate *Salmonella* competition and survival in the presence of other bacterial strains associated with food processing environments such as *Pseudomonas* [70]. However the major constraint of the CBR method is the limitation of 1 strain per experiment which may last for the duration of 1-2 weeks, depending on conditions. Moreover, in order to confirm repeatability and validate findings it is best practice that the results are reproduced on a number or repeated experiments which also adds to the length of time needed to attain results. The cost associated with the use of multiple reactors and the labour intensive method also limits the number of reactors that can be maintained simultaneously.

Furthermore, a second area of interest would be to perform a complete genomic analysis of the *Salmonella* strains of interest. As discussed in chapter 1, there are a number of key genomic cues involved in *Salmonella* biofilm development. This has been studied and reviewed to a great extent elsewhere [28]. However, similar to what has been reported in this research, relating to biofilm substrata and reactor models, the majority of the research has been performed on a limited number of strains, serovars or using a basic microtitre plate model. While the microtitre based model has advantages of high-throughput assessment of a large number of strains simultaneously, it has clearly been demonstrated in this thesis that surface substrata and biofilm conditions such as duration of biofilm development can have a major impact on biofilm density. Ideally, if there were no limitations on time and cost, a full genomic analysis through microarray

studies may solve some of the questions surrounding this topic. Whole genome sequencing of the SAGOXB.0066 strain and other *S.* Agona strains was undertaken during this research by collaborators (Professor Mark Achtman *et al.* University College Cork [185]). All *S*. Agona strains involved in this research were referred to the whole genome research group at UCC. However, due to time constrains the project did not assess the sequences generated specifically for biofilm related genes.

It would have been of interest to investigate if there were any important differences in genes up/down regulated that differentiated the density of biofilm formed by particular strains over an extended period of time. For example, the two *S*. Agona strains had notable different biofilm density levels after 168 hours biofilm formation. RNA samples were isolated from each biofilm run using Total RNA freeze and archived at -80°C following the recommended conditions. Due to time constrains, the achieved samples were not used for microarray studies, as initially desired.

Moreover, it would have been of use to identify the main biofilm properties that lead to resistance to the disinfectant agents, such as particular proteins contained in the EPS and if the EPS was dependent on the biofilm substratum.

Most food processing environments, a biofilm may consist of multiple species of organisms. As a result it may be of interest to consider the inclusion of multiple organisms in laboratory based biofilm studies. Examining *S. enterica* biofilm density when developed in a mixed biofilm was also discussed for inclusion in this project. However, due to time constraints, it was determined that it may be more appropriate to undertake mixed species biofilm after single species biofilm studies are

better understood. The research presented in this thesis provides comprehensive investigation into the density of *S. enterica* biofilm formed on contact surfaces, the density of biofilm formed after an extensive period of time and the efficacy of disinfectant agents against an established *S. enterica* biofilm. Moreover, the research presented in this thesis provides a clearer description on the level of repeatability using biofilm development models and the caution needed when suggesting significant differences between strains based on a limited number of strains, biofilm substrata or repeated measures.

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Appendices

Appendix 1— List of Reagents

Chapter	Reagent	Supplier	Product code
2/3/4	Micro90	Sigma Aldrich	Z81565
2/3/4	100% Ethanol	Sigma Aldrich	459836
2/3/4	Sterile H_20	B.Braun	0082479
2/3/4	Tryptic Soya Broth	Sigma Aldrich	T8907
2/3/4	Tryptic Soya Agar	Sigma Aldrich	22091
2/3/4	Phosphate Buffered Saline	Sigma Aldrich	P5368
2/3	5% Glutaraldehyde	Agar Scientific	R1020
2/3	Paraformaldehyde powder	Sigma Aldrich	15827
2/3	Sodium Cacodylate	Sigma Aldrich	20840
2/3	Hydrochloric Acid	Sigma Aldrich	H1758
2/3	Osmium Tetroxide	Sigma Aldrich	R1024
2/3	Ethanol Series	Sigma Aldrich	459844
2/3	Hexamthyl Disilizane	Agar Scientific	R1228
2/3	Purified H_20 (18Ω)	N/A	
2/3	Silver paint adhesive	Agar Scientific	G302
4/5	Sodium Hypochlorite	Sigma Aldrich	425044
4/5	Sodium Hydroxide	Sigma Aldrich	30620
4/5	Benzalkonium Chloride	Sigma Aldrich	12060
4/5	D/E Neutralizing Broth	Becton Dickinson	281910
4/5	Sodium Thiosulfate	Sigma Aldrich	217263
5	Bacto-Tryptone	Sigma Aldrich	T7293
5	Yeast Extract	Sigma Aldrich	Y4250
5	Agar	Sigma Aldrich	17221
5	Congo Red	Sigma Aldrich	C6277
5	Coomassie Blue Brilliant R	Sigma Aldrich	B7920
5	1% Crystal Violet stain	Sigma Aldrich	Vv5265

Appendix 1 –List of Equipment

Chapter	Equipment	Supplier	Manufacturer	Product code
2-5	Glycerol Beads	LIP Diagnostics	Technical Services	Protect TS/70
2-5	Petri Dishes	Sarstedt		82.1473
1/2/3	Spreaders	Sarstedt		86.1569.005
1/2/3	10ml Glass Universals	LIP Diagnostics	International Scientific Supplies Limited	GMT005.P18
1/2/3	CDC Biofilm Reactors	BioSurface Technologies		CRB-90-2
1/2/3	10 Litre Carboys	Thermofisher Scientific	Nalgene	ASP-750-010
1/2/3	Large tip filters (0.2µm)	Thermofisher Scientific	Nalgene	Fdm-316-060w
1/2/3	Small tip Filters (0.2µm)	Thermofisher Scientific	Nalgene	
1/2/3	Plastic tubing 16mm	Thermofisher Scientific	MasterFlex	L/S 16 (06424-16)
1/2/3	Plastic tubing 14mm	Thermofisher Scientific	MasterFlex	L/s 18 (06424-18)
1/2/3	Connection Brackets	Thermofisher Scientific		
1/2/3	Bulkhead Fitting	Thermofisher Scientific		Btk-686-050t
1/2/3	Nylon barbed reduce connector	Thermofisher Scientific		30622-28
1/2/3	Flow Cell Breakers	BioSurface Technologies		
1/2/3	Peristaltic pump	Thermofisher Scientific	MasterFlex	07553-87
1/2/3	pump head	Thermofisher Scientific	MasterFlex	77200-60
1/2/3	Magnetic Stirrers	Thermofisher Scientific	IKA RCT	FAB-OFF-210J
1/2/3	Coupons: Borosilicate glass	BioSurface Technologies		RD128-GL
1/2/3	Coupons: Stainless steel	BioSurface Technologies		RD128-316
1/2/3	Coupons: Polycarbonate	BioSurface Technologies		RD-128-PC
1/2/3	Coupons: Glazed Tile	BioSurface Technologies		RD128-GT
1/2/3	Coupons: Concrete	BioSurface Technologies		RD128-CC
1/2/3	Forceps and set screw	BioSurface Technologies		

Appendix 1 – List of Equipment- Continued

Chapter	Equipment	Supplier	Manufacturer	Product code
1/2/3	24-well plates	Sarstedt		83.1836.500
1/2/3	Bio Assay Dishes		Nunc	40835
1/2/3	Sonicator		NEY DENTAL INC	ULTRAsonik™ Cleaner Model 28X
1/2	Gold Sputter	Emscope SC500		
1/2	Microscope	Hitachi S52600N		
1/2	Carbon Tape adhesive	Agar Scientific		
1/2	Metal Stubs	Agar Scientific		
4	Microtitre Plates flat-bottom 96 well plate	Sarstedt		DIS-984-170X untreated
4	Turbidity Meter	Medical Supplies Company Limited	Siemens	Microscan Turbidity Meter
4	Plate Reader	Dynex Tecnologies		Best 2000
4	Plate reader technology	Revelation Software		Version 5.19
1-4	Statistical Package for Social Sciences (SPSS)	IBM Corporation		Version 20.0

Appendix 2

Table 1: The optical density of biofilm formed on the surface

Strain No	Strain	well	1st OD Read	2nd OD Read	Difference in OD	Mean OD
27853	*P. aeruginosa*	1	0.148	0.229	-0.081	0.197
27853	*P. aeruginosa*	2	0.234	0.216	0.018	
27853	*P. aeruginosa*	3	0.179	0.176	0.003	
S08-0601	*S.* Agona Outbreak	1	0.344	0.218	0.126	0.453
S08-0601	*S.* Agona Outbreak	2	1.641	0.272	1.369	
S08-0601	*S.* Agona Outbreak	3	0.141	0.100	0.041	
S09-0494	*S.* Agona Outbreak	1	0.339	0.504	-0.165	0.265
S09-0494	*S.* Agona Outbreak	2	0.260	0.245	0.015	
S09-0494	*S.* Agona Outbreak	3	0.130	0.113	0.017	
S09-0046	*S.* Agona variant	1	0.157	0.179	-0.022	0.111
S09-0046	*S.* Agona variant	2	0.091	0.107	-0.016	
S09-0046	*S.* Agona variant	3	0.057	0.074	-0.017	
S09-0371	*S.* Agona	1	0.870	0.603	0.267	0.66
S09-0371	*S.* Agona	2	1.119	1.112	0.007	
S09-0371	*S.* Agona	3	0.142	0.115	0.027	
S09-0479	*S.* Agona	1	0.119	0.109	0.010	0.125
S09-0479	*S.* Agona	2	0.074	0.072	0.002	
S09-0479	*S.* Agona	3	0.209	0.168	0.041	
SL483	*S.* Agona	1	0.473	0.634	-0.161	0.609
SL483	*S.* Agona	2	0.589	0.566	0.023	
SL483	*S.* Agona	3	0.819	0.571	0.248	
S09-0419	*S.* Typhimurium	1	0.085	0.082	0.003	0.085
S09-0419	*S.* Typhimurium	2	0.070	0.086	-0.016	
S09-0419	*S.* Typhimurium	3	0.099	0.089	0.010	
S08-0408	*S.* Typhimurium	1	0.346	0.27	0.076	0.234
S08-0408	*S.* Typhimurium	2	0.231	0.34	-0.109	
S08-0408	*S.* Typhimurium	3	0.111	0.104	0.007	
SL1344	*S.* Typhimurium	1	0.238	0.271	-0.033	0.391
SL1344	*S.* Typhimurium	2	0.147	0.15	-0.003	
SL1344	*S.* Typhimurium	3	0.872	0.666	0.206	
LT2	*S.* Typhimurium	1	0.103	0.112	-0.009	0.182
LT2	*S.* Typhimurium	2	0.477	0.112	0.365	
LT2	*S.* Typhimurium	3	0.153	0.135	0.018	
S09-0717	*S.* Enteritidis	1	0.352	0.286	0.066	0.301
S09-0717	*S.* Enteritidis	2	0.280	0.307	-0.027	
S09-0717	*S.* Enteritidis	3	0.271	0.309	-0.038	
S09-0004	*S.* Enteritidis	1	0.233	0.413	-0.18	0.232
S09-0004	*S.* Enteritidis	2	0.119	0.109	0.010	
S09-0004	*S.* Enteritidis	3	0.214	0.305	-0.091	

Colour index

High outlier
Low outlier
Large Difference in OD

Appendix 2 - Table 1: displays the optical density biofilm formed in 3 replicate wells for each for 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 6 *Salmonella* Typhimurium strains and 2 *Salmonella* Enteritidis strains examined. The optical density was measured at 590 wavelengths (OD_{590}). The measurement of OD_{590} was repeated on the same plate twice to examine the repeatability of measurement of OD_{590} values. Each strain was examined in triplicate wells in the microtitre plate. The high outliers are denoted by yellow coloured cell and the low outliers are denoted by blue coloured cell. The difference between the repeated reads was calculated by subtracting the 1st read from the 2nd read of OD_{590}. A large difference in the reads is denoted by a pink coloured cell. The mean of all 6 observations for each strain was also calculated. These mean calculations are based on the biofilm density formed over 48 hours at 22°C.

Appendix 2 - Table 2: 48 Hour biofilm at 22°C

Strain No	Strain	well	Run 1	Run 2	Run 3	Mean of all 9 OD
27853	*P. aeruginosa*	1	0.353	0.114	0.148	0.184
27853	*P. aeruginosa*	2	0.175	0.14	0.234	
27853	*P. aeruginosa*	3	0.169	0.142	0.179	
S08-0601	*S.* Agona Outbreak	1	0.385	0.425	0.344	0.815
S08-0601	*S.* Agona Outbreak	2	1.507	1.852	1.641	
S08-0601	*S.* Agona Outbreak	3	0.569	0.47	0.141	
S09-0494	*S.* Agona Outbreak	1	0.224	0.186	0.339	0.204
S09-0494	*S.* Agona Outbreak	2	0.249	0.157	0.260	
S09-0494	*S.* Agona Outbreak	3	0.149	0.145	0.130	
S09-0046	*S.* Agona variant	1	0.508	0.206	0.157	0.386
S09-0046	*S.* Agona variant	2	0.308	0.959	0.091	
S09-0046	*S.* Agona variant	3	0.248	0.936	0.057	
S09-0371	*S.* Agona	1	0.344	0.214	0.870	0.450
S09-0371	*S.* Agona	2	0.194	0.752	1.119	
S09-0371	*S.* Agona	3	0.165	0.251	0.142	
S09-0479	*S.* Agona	1	0.586	0.147	0.119	0.351
S09-0479	*S.* Agona	2	0.240	0.391	0.074	
S09-0479	*S.* Agona	3	0.639	0.755	0.209	
SL483	*S.* Agona	1	0.323	0.224	0.473	0.405
SL483	*S.* Agona	2	0.398	0.291	0.589	
SL483	*S.* Agona	3	0.232	0.298	0.819	
S09-0419	*S.* Typhimurium	1	0.198	0.114	0.085	0.245
S09-0419	*S.* Typhimurium	2	0.245	0.951	0.070	
S09-0419	*S.* Typhimurium	3	0.313	0.126	0.099	
S08-0408	*S.* Typhimurium	1	0.356	0.107	0.346	0.224
S08-0408	*S.* Typhimurium	2	0.314	0.235	0.231	
S08-0408	*S.* Typhimurium	3	0.250	0.066	0.111	
SL1344	*S.* Typhimurium	1	0.939	0.199	0.238	0.723
SL1344	*S.* Typhimurium	2	1.066	1.632	0.147	
SL1344	*S.* Typhimurium	3	0.439	0.973	0.872	
LT2	*S.* Typhimurium	1	0.246	0.170	0.103	0.204
LT2	*S.* Typhimurium	2	0.191	0.198	0.477	
LT2	*S.* Typhimurium	3	0.202	0.095	0.153	
S09-0717	*S.* Enteritidis	1	0.514	0.646	0.352	0.285
S09-0717	*S.* Enteritidis	2	0.122	0.151	0.28	
S09-0717	*S.* Enteritidis	3	0.113	0.115	0.271	
S09-0004	*S.* Enteritidis	1	0.174	1.020	0.233	0.260
S09-0004	*S.* Enteritidis	2	0.086	0.194	0.119	
S09-0004	*S.* Enteritidis	3	0.137	0.166	0.214	

Colour index

High outlier
Low outlier

Appendix 2 - Table 2 displays the optical density biofilm formed 3 replicate wells for each for 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 6 *S.* Typhimurium strains and 2 *S.* Enteritidis strains examined. The optical density was measured at 590 wavelengths (OD_{590}). Each strain was examined in triplicate wells in the microtitre plate (wells 1-3). The experiment was performed on three occasions (Run 1-3). The high outliers are denoted by yellow coloured cell and the low outliers are denoted by blue coloured cell. The mean of all 9 observations for each strain was also calculated. These mean calculations are based on the biofilm density formed over 48 hours at 37°C.

Appendix 2 - Table 3: 48 hour biofilm at 37°C

Strain No	Strain	well	Run 1	Run 2	Run 3	Mean of all 9 OD
27853	*P. aeruginosa*	1	0.172	0.545	0.128	0.540
27853	*P. aeruginosa*	2	0.668	0.351	0.415	
27853	*P. aeruginosa*	3	1.348	0.285	0.947	
S08-0601	*S.* Agona Outbreak	1	0.099	0.083	1.505	0.291
S08-0601	*S.* Agona Outbreak	2	0.072	0.057	0.309	
S08-0601	*S.* Agona Outbreak	3	0.083	0.29	0.117	
S09-0494	*S.* Agona Outbreak	1	0.094	0.216	0.226	0.227
S09-0494	*S.* Agona Outbreak	2	0.194	0.262	0.428	
S09-0494	*S.* Agona Outbreak	3	0.175	0.142	0.305	
S09-0046	*S.* Agona variant	1	0.07	0.145	0.254	0.104
S09-0046	*S.* Agona variant	2	0.064	0.083	0.107	
S09-0046	*S.* Agona variant	3	0.058	0.059	0.099	
S09-0371	*S.* Agona	1	0.068	0.179	0.125	0.091
S09-0371	*S.* Agona	2	0.075	0.072	0.057	
S09-0371	*S.* Agona	3	0.081	0.098	0.066	
S09-0479	*S.* Agona	1	0.042	0.111	0.058	0.187
S09-0479	*S.* Agona	2	0.286	0.255	0.146	
S09-0479	*S.* Agona	3	0.379	0.334	0.069	
SL483	*S.* Agona	1	0.109	0.143	0.088	0.149
SL483	*S.* Agona	2	0.097	0.29	0.052	
SL483	*S.* Agona	3	0.265	0.121	0.177	
S09-0419	*S.* Typhimurium	1	0.062	0.202	0.206	0.144
S09-0419	*S.* Typhimurium	2	0.062	0.07	0.074	
S09-0419	*S.* Typhimurium	3	0.096	0.316	0.21	
S08-0408	*S.* Typhimurium	1	0.147	0.095	0.276	0.114
S08-0408	*S.* Typhimurium	2	0.065	0.047	0.096	
S08-0408	*S.* Typhimurium	3	0.046	0.073	0.179	
SL1344	*S.* Typhimurium	1	0.108	0.074	0.172	0.294
SL1344	*S.* Typhimurium	2	0.139	0.134	1.651	
SL1344	*S.* Typhimurium	3	0.094	0.133	0.144	
LT2	*S.* Typhimurium	1	0.224	0.19	0.091	0.183
LT2	*S.* Typhimurium	2	0.35	0.131	0.093	
LT2	*S.* Typhimurium	3	0.186	0.172	0.211	
S09-0717	*S.* Enteritidis	1	0.145	0.326	0.056	0.159
S09-0717	*S.* Enteritidis	2	0.136	0.359	0.043	
S09-0717	*S.* Enteritidis	3	0.078	0.232	0.055	
S09-0004	*S.* Enteritidis	1	0.108	0.247	0.051	0.103
S09-0004	*S.* Enteritidis	2	0.059	0.147	0.067	
S09-0004	*S.* Enteritidis	3	0.072	0.12	0.056	

Colour index

High outlier
Low outlier

Appendix 2 - Table 3 displays the optical density biofilm formed 3 replicate wells for each for 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 6 *S.* Typhimurium strains and 2 *S.* Enteritidis strains examined. The optical density was measured at 590 wavelengths (OD_{590}). Each strain was examined in triplicate wells in the microtitre plate (wells 1-3). The experiment was performed on three occasions (Run 1-3). The high outliers are denoted by yellow coloured cell and the low outliers are denoted by blue coloured cell. The mean of all 9 observations for each strain was also calculated. These mean calculations are based on the biofilm density formed over 48 hours at 37°C.

Appendix 2 – Table 4: 168 hour biofilm at 22°C

Strain No	Strain	well	Run 1	Run 2	Run 3	Mean of all 9 OD readings
27853	*P. aeruginosa*	1	0.339	0.209	0.453	0.329
27853	*P. aeruginosa*	2	0.306	0.234	0.292	
27853	*P. aeruginosa*	3	0.31	0.448	0.371	
S08-0601	*S.* Agona Outbreak	1	0.239	0.089	0.351	0.193
S08-0601	*S.* Agona Outbreak	2	0.179	0.059	0.37	
S08-0601	*S.* Agona Outbreak	3	0.126	0.07	0.257	
S09-0494	*S.* Agona Outbreak	1	0.193	0.103	1.235	0.302
S09-0494	*S.* Agona Outbreak	2	0.232	0.079	0.349	
S09-0494	*S.* Agona Outbreak	3	0.166	0.064	0.299	
S09-0046	*S.* Agona variant	1	0.16	0.262	0.315	0.280
S09-0046	*S.* Agona variant	2	0.105	0.062	0.288	
S09-0046	*S.* Agona variant	3	0.099	0.346	0.885	
S09-0371	*S.* Agona	1	0.067	0.07	0.303	0.360
S09-0371	*S.* Agona	2	0.096	0.087	2.226	
S09-0371	*S.* Agona	3	0.086	0.062	0.247	
S09-0479	*S.* Agona	1	0.237	0.099	0.964	0.308
S09-0479	*S.* Agona	2	0.177	0.11	0.542	
S09-0479	*S.* Agona	3	0.188	0.12	0.333	
SL483	*S.* Agona	1	0.122	0.143	0.156	0.233
SL483	*S.* Agona	2	0.338	0.088	0.322	
SL483	*S.* Agona	3	0.626	0.133	0.17	
S09-0419	*S.* Typhimurium	1	0.462	0.245	0.626	0.368
S09-0419	*S.* Typhimurium	2	0.379	0.16	0.303	
S09-0419	*S.* Typhimurium	3	0.204	0.416	0.515	
S08-0408	*S.* Typhimurium	1	0.504	0.101	0.542	0.251
S08-0408	*S.* Typhimurium	2	0.144	0.246	0.269	
S08-0408	*S.* Typhimurium	3	0.11	0.095	0.247	
SL1344	*S.* Typhimurium	1	0.138	0.111	0.168	0.173
SL1344	*S.* Typhimurium	2	0.116	0.189	0.185	
SL1344	*S.* Typhimurium	3	0.368	0.163	0.123	
LT2	*S.* Typhimurium	1	0.225	0.244	0.122	0.166
LT2	*S.* Typhimurium	2	0.282	0.044	0.143	
LT2	*S.* Typhimurium	3	0.245	0.043	0.145	
S09-0717	*S.* Enteritidis	1	0.189	0.043	0.318	0.199
S09-0717	*S.* Enteritidis	2	0.155	0.044	0.306	
S09-0717	*S.* Enteritidis	3	0.168	0.133	0.439	
S09-0004	*S.* Enteritidis	1	0.161	0.058	0.335	0.173
S09-0004	*S.* Enteritidis	2	0.207	0.075	0.206	
S09-0004	*S.* Enteritidis	3	0.225	0.075	0.219	

Colour index

High outlier
Low outlier

Appendix 2 - Table 4 displays the optical density biofilm formed 3 replicate wells for each for 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 6 *S.* Typhimurium strains and 2 *S.* Enteritidis strains examined. The optical density was measured at 590 wavelengths (OD_{590}). Each strain was examined in triplicate wells in the microtitre plate (wells 1-3). The experiment was performed on three occasions (Run 1-3). The high outliers are denoted by yellow coloured cell and the low outliers are denoted by blue coloured cell. The mean of all 9 observations for each strain was also calculated. These mean calculations are based on the biofilm density formed over 168 hours at 22°C.

Appendix 2 - Table 5: 168 hour biofilm at 37°C

Strain No	Strain	Well	Run 1	Run 2	Run 3	Mean of all 9 OD readings
27853	*P. aeruginosa*	1	0.675	1.143	0.989	0.788
27853	*P. aeruginosa*	2	0.646	2.031	0.286	
27853	*P. aeruginosa*	3	0.477	0.244	0.598	
S08-0601	*S.* Agona Outbreak	1	0.097	0.072	0.995	0.699
S08-0601	*S.* Agona Outbreak	2	0.044	0.198	2.516	
S08-0601	*S.* Agona Outbreak	3	0.041	0.603	1.726	
S09-0494	*S.* Agona Outbreak	1	0.054	0.109	3.358	1.200
S09-0494	*S.* Agona Outbreak	2	0.282	0.102	1.94	
S09-0494	*S.* Agona Outbreak	3	1.013	0.754	3.189	
S09-0046	*S.* Agona variant	1	0.041	0.183	2.441	0.334
S09-0046	*S.* Agona variant	2	0.041	0.037	0.076	
S09-0046	*S.* Agona variant	3	0.036	0.057	0.098	
S09-0371	*S.* Agona	1	0.045	0.041	0.15	0.234
S09-0371	*S.* Agona	2	0.583	0.286	0.048	
S09-0371	*S.* Agona	3	0.168	0.244	0.543	
S09-0479	*S.* Agona	1	0.122	0.111	0.304	0.146
S09-0479	*S.* Agona	2	0.146	0.064	0.104	
S09-0479	*S.* Agona	3	0.048	0.044	0.369	
SL483	*S.* Agona	1	0.253	0.079	0.217	1.162
SL483	*S.* Agona	2	0.797	0.16	0.245	
SL483	*S.* Agona	3	3.101	2.392	3.218	
S09-0419	*S.* Typhimurium	1	0.09	0.426	0.064	0.147
S09-0419	*S.* Typhimurium	2	0.057	0.047	0.087	
S09-0419	*S.* Typhimurium	3	0.044	0.454	0.055	
S08-0408	*S.* Typhimurium	1	0.046	0.041	0.123	0.095
S08-0408	*S.* Typhimurium	2	0.056	0.046	0.108	
S08-0408	*S.* Typhimurium	3	0.172	0.044	0.216	
SL1344	*S.* Typhimurium	1	0.071	0.047	0.142	0.099
SL1344	*S.* Typhimurium	2	0.036	0.048	0.154	
SL1344	*S.* Typhimurium	3	0.058	0.105	0.226	
LT2	*S.* Typhimurium	1	0.083	0.197	0.076	0.519
LT2	*S.* Typhimurium	2	1.259	0.046	0.119	
LT2	*S.* Typhimurium	3	2.041	0.555	0.294	
S09-0717	*S.* Enteritidis	1	1.239	0.489	0.069	0.294
S09-0717	*S.* Enteritidis	2	0.205	0.171	0.039	
S09-0717	*S.* Enteritidis	3	0.081	0.235	0.12	
S09-0004	*S.* Enteritidis	1	0.084	0.292	0.056	0.235
S09-0004	*S.* Enteritidis	2	0.889	0.051	0.253	
S09-0004	*S.* Enteritidis	3	0.111	0.161	0.215	

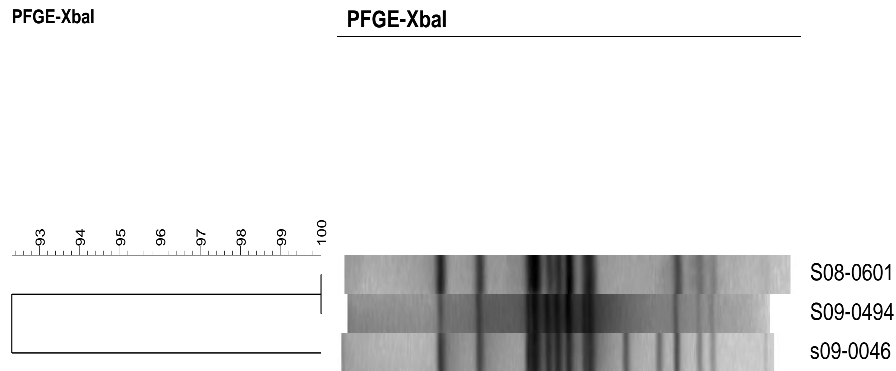
Colour index

High outlier
Low outlier

Appendix 2 - Table 5 displays the optical density biofilm formed 3 replicate wells for each for 6 *Salmonella enterica* subspecies *enterica* serovar Agona strains, 6 *S.* Typhimurium strains and 2 *S.* Enteritidis strains examined. The optical density was measured at 590 wavelengths (OD_{590}). Each strain was examined in triplicate wells in the microtitre plate (wells 1-3). The experiment was performed on three occasions (Run 1-3). The high outliers are denoted by yellow coloured cell and the low outliers are denoted by blue coloured cell. The mean of all 9 observations for each strain was also calculated. These mean calculations are based on the biofilm density formed over 168 hours at 37°C.

Appendices 3

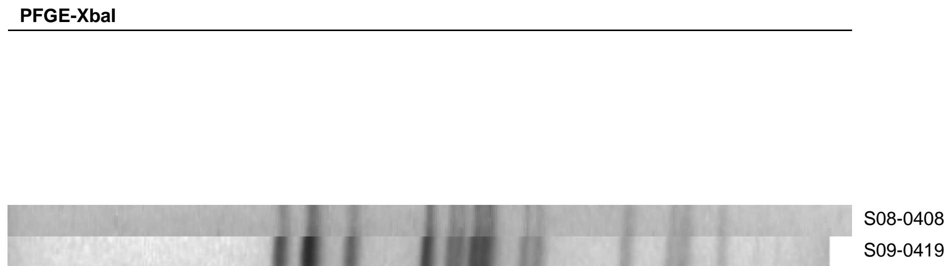
Appendix 3 - Figure 1: PFGE Image of *S*. Agona SAGOXB.0066 Outbreak strains



Appendix 3 - Figure 1: displays the pulse field gel electrophoresis (PFGE) pattern of the three *Salmonella enterica* subspecies *enterica* serovar Agona strains involved in the SAGOXB.0066 outbreak. The image indicates that the two strains S08-0601 and S09-0494 are 100% indistinguishable by PFGE analysis. The third strain (the variant strain S09-0046) was 92% similar by PFGE and had to additional bands in comparison to the orignal strains related to the outbreak.

The image was provided by the National *Salmonella Shigella* and *Listeria* Reference Laboratory as PFGE was not part of this PhD research.

Appendix 3-Figure 2: PFGE Image of *S*. Typhimurium and monophasic STYMXB.131 strains



Appendix 3 - Figure 2: displays the pulse field gel electrophoresis (PFGE) pattern of the two *Salmonella enterica* subspecies *enterica* serovar Typhimurium strains indentified as STYMXB.0131. The image indicates that the two strains S08-0408 and S09-0419 are 100% indistinguishable by PFGE analysis, despite the S09-0419 not displaying H antigens (monophasic).

The image was provided by the National *Salmonella Shigella* and *Listeria* Reference Laboratory as PFGE analysis was not part of this PhD thesis project.

Dissemination of Research

1. Lead Authorship - Peer Reviewed publications

Title: *Salmonella enterica* Biofilm Formation and Density in the Centers for Disease Control and Prevention's Biofilm Reactor Model Is Related to Serovar and Substratum
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Journal: Journal of Food Protection April 2013
Volume: 76 **Issue:** 4 **Pages:** 662-667
DOI: 10.4315/0362-028X.JFP-12-303

Title: Bactericidal activity of disinfectant against *Salmonella enterica* biofilm is dependent on biofilm age.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Process: Currently under review.

2. Lead Authorship - Conference Proceedings

Oral Presentation at the 2013 Environ research Conference
Title: *Salmonella enterica* can readily form a biofilm in the environment and is highly resistant to eradication with chemical disinfectants.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: NUI, Galway.

Poster Presentation at Global Food Safety conference – October 2012.
Title: Biofilm formation a threat to public safety?
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: Dublin, Ireland.

Poster Presentation at RAMI Biomedical Sciences Section Annual Meeting –July 2012.
Title: How to kill a *Salmonella* biofilm on contact surfaces.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: NUI, Galway.

Poster Presentation Environmental Research Institute – June 2012.
Title: Prevention of Food-borne Salmonellosis: The challenge of killing a biofilm.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: NUI, Galway.

Oral Presentation at School of Medicine Annual Research Day – May 2012.
Title: Biofilm age can impact the efficacy of disinfectants.
Location: NUI, Galway.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Awarded second prize for best presentation of research.

Poster Presentation at School of Medicine Annual Research Day – May 2012.
Title: Killing an established biofilm.
Location: NUI, Galway.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.

Poster Presentation at Federation of Infections Society— November 2011.
Title: *Salmonella enterica* biofilm: older biofilms are different.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: Manchester, UK.

Oral Presentation at British Society for Antimicrobial Chemotherapy – November 2011.
Antimicrobial Resistance Mechanisms Workshop.
Title: How to kill a *Salmonella* Biofilm.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: Birmingham, UK.
Received full sponsorship through BSAC Student sponsorship programme to travel and present my work at the workshop.

Poster Presentation at American Society for Microbiology – May 2011.
Title: Variation in *Salmonella enterica* Serovars Ability to Establish a Biofilm.
Location: New Orleans, Louisiana, USA.
Received a travel grant worth $500, based on the ASM committee review of abstracts.

Oral Presentation at Society of General Microbiology – April 2011.
Title: *Salmonella enterica* serovar Enteritidis biofilm formation on 5 surfaces found in food processing environments.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: Queens University Belfast, UK.

Poster Presentation at Society for General Microbiology – April 2011.
Title: Differences in the biofilm properties of laboratory adapted and recent isolates of *Salmonella enterica*.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: International Conference Centre, Harrogate, UK.

Oral Presentation at Society for General Microbiology — April 2011.
Title: The ability of *Salmonella enterica* to form a biofilm on food contact surfaces.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: University of Nottingham, UK.
Received a travel grant worth £320 pounds for attending the conference.

Oral Presentation at International Association of Food Protection – June 2010.
Title: Differences in the ability of contact materials to support *Salmonella enterica* biofilm formation.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: University College Dublin, Ireland.

Poster Presentation at Society for General Microbiology – April 2010.
Title: Establishing a Laboratory model of *Salmonella enterica* biofilm.
Authors: M. Corcoran, D. Morris, N. De Lappe, J. O'Connor, P. Lalor, P. Dockery and M. Cormican.
Location: NUI, Galway.