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Persistent contamination of *Salmonella, Campylobacter, Escherichia coli and Staphylococcus aureus* at a Broiler Farm in New Zealand

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Abstract

The public demand for poultry products has increased over the years due to their health benefits and relatively low cost. Intensive production of poultry in broiler farms gives an opportunity for contamination of the birds, thus creating potential foodborne hazards to consumers. Foodborne cases are therefore extensively monitored to implement mitigating strategies to control the outbreaks. Therefore, the main aim of this project was to determine the prevalence and microbial loads of contaminating *Campylobacter* spp., *Salmonella* spp., *S. aureus* and *E. coli*, in different locations of four broiler sheds at a selected poultry farm in Auckland New Zealand. Standard microbiological methods and multiplex quantitative polymerase chain reaction (qPCR) were used in the analyses. Swab samples were collected in three cycles from March 2016 to June 2016. During each cycle of the cleaning and disinfection regime, 248 swab samples were collected from feeders, feed loaders, drinkers, fans, vents, annex floor, and wall crevices to determine the extent of contamination before cleaning and after disinfection. The collected samples (n = 744) were analysed for the presence of *Salmonella* spp. and *Campylobacter* spp. using standard microbiological methods. Suspected isolates of *Salmonella* spp. were confirmed by latex agglutination test, whilst *Campylobacter* spp. was confirmed by both latex agglutination and oxidase tests. The swab samples were also analysed for viable *S. aureus* and *E. coli* cell counts using Petrifilm™ plates. Multiplex qPCR was developed and validated to enumerate *Salmonella* spp. and *Campylobacter* spp. positive samples.

Results of this study showed that all collected samples were contaminated with *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* before performing cleaning. After disinfection, different areas of the shed were still contaminated, posing real danger for infection of the new flock. Crevices and drinkers were the most contaminated areas after disinfection. Organic matter that accumulates in crevices and drinkers during rearing are likely to protect pathogens against disinfectants, which may then contribute to residual contamination and biofilm formation. The ventilation system of the farm was also heavily contaminated**.** After disinfection, dusts were trapped between the wires of the ventilation screen, making air vents a potential source of contamination in poultry sheds. Feed loaders had higher contamination rates than feeders, even though it was elevated, away from direct contact to birds. When the ventilation system was open, contaminated dusts settle into various areas of the shed, thereby increasing contamination levels before cleaning, thus affecting the efficacy of the disinfectant used. Meanwhile, fans and the annex were less contaminated, indicating that the cleaning regime could effectively disinfect these areas. However, results showed that microbial concentration in the annex was higher after disinfection. This was probably caused by the introduction of pathogens from the outside environment, highlighting the importance of erecting hygiene barriers before entering the main shed.

Multiplex qPCR is an important quantification tool due to its ability to detect, identify and quantify multiple pathogens in one assay. The standard curves generated from inoculated samples determined the detection limit to be 3.24 - 8.24 Log_{10} CFU/mL for *Salmonella* spp., and 2.97 - 7.97 Log_{10} CFU/mL for *Campylobacter* spp. respectively. The agreement of results using the standard and qPCR methods was investigated by comparing *S. aureus* counts obtained from100 environmental samples through Bland-Altman analysis. The two methods showed agreement, but the qPCR was limited to the detection of *S. aureus* from 3.5 to 6 Log_{10} CFU/mL. The concentration of *Salmonella* spp. and *Campylobacter* spp. enumerated by multiplex qPCR, had no significant difference between the mean counts of each location before cleaning and after disinfection. Concentration of *Salmonella* spp. and *Campylobacter* spp. in the samples subjected to analysis by qPCR post-disinfection, were below the detection limit of the method. However, the qPCR method may be suitable for analysis of samples collected before cleaning. Pre- enrichment of samples analysed post-disinfection is recommended to improve the detection and enumeration of *Salmonella* spp. and *Campylobacter* spp. by qPCR analysis.

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Abbreviation

AAAAI	=	The American Academy of Allergy, Asthma & Immunology
ACMF	=	Australian Chicken Meat Federation
A/E	=	Attaching and effacing
AOAC	=	Association of Official Analytical Chemists International
APC	=	Antigen Presenting Cells
BAM	=	Bacteriological Analytical Manual
BHI	=	Brain Heart Infusion
BPW	=	Buffered Peptone Water
CFU	=	Colony Forming Unit
DNA	=	Deoxyribonucleic Acid
EIEC	=	Enteroinvasive *E. coli*
EHEC	=	Enterohemorrhagic *E. coli*
EPEC	=	Enteropathogenic *E. coli*
ESR	=	Environmental Science and Research
ETEC	=	Enterotoxigenic *E. coli*
FDA	=	Food and Drug Administration
GBS	=	Guillain-Barre syndrome
LEE	=	Locus of Enterocyte Effacement
MBG	=	Modified Brilliant Green
mCCDA	=	Modified Charcoal-Cefoperazone-Deoxycholate
MGB	=	Minor Groove Binder
MHC	=	Major Histocompatibility Complex
MPI	=	Ministry of Primary Industry
MPN	=	Most Probable Number
MRSA	=	Methicillin Resistant *S. aureus*
NaCl	=	Sodium Chloride
NCTC	=	National Collection of Type Cultures
NFQ	=	Non-Fluorescent Quencher
PCP2a	=	Penicillin Binding Proteins
PCR	=	Polymerase Chain Reaction
qPCR	=	Quantitative Polymerase Chain Reaction

rRNA	=	Ribosomal Ribonucleic Acid
RVS	=	Rappaport Vassiliadis Soya
SE	=	Staphylococcal Enterotoxins
SEA	=	Staphylococcal Enterotoxin A
SEB	=	Staphylococcal Enterotoxin B
SEC	=	Staphylococcal Enterotoxin C
SED	=	Staphylococcal Enterotoxin D
SEE	=	Staphylococcal Enterotoxin E
SEG	=	Staphylococcal Enterotoxin G
SHE	=	Staphylococcal Enterotoxin E
SEI	=	Staphylococcal Enterotoxin I
SEJ	=	Staphylococcal Enterotoxin J
SPSS	=	Statistical Package for the Social Sciences
STEC	=	Shinga toxin producing *E. coli*
TcR	=	T-cell Antigen Receptor
TAE	=	Tris-acetate-EDTA
TE	=	Tris-EDTA
UDG	=	Uracil-DNA Glycosylase
VTEC	=	Verotoxin producing *E. coli*
WHO	=	World Health Organization
XLD	=	Xylose Lysine Deoxycholate

1 Introduction

Food-borne illnesses are "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water" (WHO, 2014). There are approximately 250 food-borne diseases varying in symptoms such as nausea, abdominal cramping, diarrhoea, and vomiting (MPI, n.d). Among these diseases, the most common are caused by strains of *Campylobacter*, *Salmonella, Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (Lopez, Roos, Cressey, Horn, & Lee, 2016).

The four pathogens are important in New Zealand. In 2015, about 11 out of 19 *Campylobacter* spp., and 3 out of 18 *Salmonella* spp. outbreaks were due to foodborne transmissions, where poultry was observed to be the most common route of transmission (Lopez et al., 2016). Meanwhile, *E. coli* and *S. aureus* food poisoning had low incident rates; but monitoring their prevalence is important as they have been associated with life-threatening infections. For instance, in 2014, only 4 out of 10 *E. coli* outbreaks were due to food transmission, with consuming dairy products as their highest risk factor (Horn, Lopez, Cressey, & Pirie, 2015). Whilst in 2015, *E. coli* infections caused 17 outbreaks that were not food related (Lopez et al., 2016). Additionally, *S. aureus* food poisoning incidents in 2014 and 2015, were all due to foodborne outbreaks (3/3) (Lopez et al., 2016).

Amongst all the *Campylobacter* spp., *C. jejuni* is the most common, as it has been implicated to cause 80 % of campylobacteriosis worldwide (FDA, 2013). In New Zealand, horizontal transmission was observed to be their main infection route in poultry, where contaminated environmental reservoir contributes to the spread of pathogens (Sahin et al., 2015). *C. jejuni* can be found in various locations of the broiler farms (mainly in water, litter and handling equipment). They are observed to quickly colonize digestive tracts of poultry when infected via ingestion (MPI, n.d). Due to the way *Campylobacter* infected flocks, preventing contamination incidents has received the most attention (Cox, Berrang, & Cason, 2000; Denis, Refregier-Petton, Laisney, Ermel, & Salvat, 2001; Sahin et al., 2015).

Presently, *S.* Enteritidis and *S.* Typhimurium are the most frequently isolated *Salmonella* serotypes. They have different epidemiology compared to other *Salmonella* spp. as they cause most food poisoning events, with poultry as an important reservoir (Lofstrom,

Hintzmann, Sorensen, & Baggesen, 2015). Vertical and horizontal transmission are known as their contamination routes. For instance, *S.* Enteritidis are observed to infect poultry by vertical transmission, where they cause ovarian infections (Heyndrickx et al., 2002). Both bird and their offspring become chronic carriers when this transmission happens (Agada et al., 2014). *S.* Typhimurium however, is horizontally transmitted from contaminated environments (Heyndrickx et al., 2002). In humans, the transmission occurs due to the ingestion of food or water, which has been contaminated with faeces of infected birds or carriers (Agada et al., 2014). Diseased poultry is easily identifiable, thus can be removed and/or treated (Gantois et al., 2009). However, *Salmonella* carriers infect flocks without causing obvious symptoms, which makes infection difficult to control (Heyndrickx et al., 2002).

Among the *E. coli* serotypes, *E. coli* 0157:H7 has been recognised to cause severe pathogenic human infections. They are observed to attach and colonise the intestinal lining of a host and disrupt epithelial cell functions (Caprioli, Morabito, Brugère, & Oswald, 2005). Different strains are characterised by their production of cytotoxin, which inhibits protein synthesis in eukaryotes, and cause varying symptoms, from mild diarrhoea to haemorrhagic colitis (Beutin, 2006; Caprioli et al., 2005). *E. coli* infection has been observed to occur mainly due to contaminated food, water, milk and vegetables (Lake, Hudson, & Cressey, 2003). Previous studies suggest that their main transmission route in poultry is through vertical transmission. *E. coli* populations may decrease by two folds when they become exposed to unfavourable environmental conditions, thus demonstrating their inability to survive outside a host (Winfield & Groisman, 2003). However, horizontal transmission can still occur because pests and insects can serve as vectors for *E. coli* infection (Beutin, 2006).

S. aureus is important to understand because of their intrinsic resistance to all ß-lactam antibiotics, such as penicillin (Tokue, Shoji, Satoh, Watanabe, & Motomiya, 1992). The food poisoning incidence by this pathogen mainly occurs due to poor handling practices, and insufficient sanitisation (Pinchuk et al., 2010). Their transmission routes in poultry are not extensively studied because of their low prevalence. Nonetheless, Wendlandt et al. (2013) investigated possible transmission pathways; and concluded that *S. aureus* are

transmitted between farmers and broiler houses; Thereby infecting flocks by releasing different enterotoxins, such as *Staphylococcus* enterotoxin A (SEA), SEB and SEF (Pinchuk et al., 2010). These enterotoxins are known as super-antigens that suppress the immune response by causing T cell proliferation (T cells are known to trigger cell signalling, and act as a defence mechanism against pathogen outbreaks) in poultry(Proft & Fraser, 2003). Due to its proliferation attribute, *S. aureus* causes severe health problems.

The occurrence of food-related infections due to poultry need better control interventions and improved prevention strategies, particularly at the farm level (Callejon et al., 2015). Different areas of broiler sheds can harbor pathogens, including litter, feed, crevices and equipment (Heyndrickx et al., 2002). Since *Campylobacter* and *Salmonella* are commonly found in the avian gut, they can thrive in various sites of broiler sheds due to contaminated faeces that are shed from infected birds (MPI, 2001). *E. coli* on the other hand, can be isolated from soil and water, only when it mimics conditions found in the internal mucosa (Beutin, 2006). Whereas, *S. aureus* are mainly found in areas that are difficult to clean and sanitise (MPI, 2001). Many bio-security risks have been identified in the New Zealand poultry industry, and many mitigation strategies have already been established (Geale et al., 2006).

Microbiological analysis has been an important part of microbial safety management in the food chain (Chapela, Garrido-Maestu, Cabado, & Yildiz, 2015; Chen, Tang, Liu, Cai, & Bai, 2012). Monitoring and controlling foodborne pathogens were traditionally carried out by standard methods that are based on culture-dependent techniques, including standard biochemical identifications. However, qPCR has become an important quantification tool in several fields of biological research due to its ability to rapidly detect, identify and quantify pathogens or beneficial bacteria (Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011).

1.1 Aim and Objectives

The aim of this project was to determine the prevalence and microbial loads of contaminating *Campylobacter*, *Salmonella, E. coli and S. aureus* from different locations of a broiler farm. The specific objectives of the study were to:

- Determine the prevalence and the level of contamination in poultry sheds before cleaning and after disinfection;
- Identify sources of contamination at the poultry farm; and,
- Develop multiplex qPCR as a rapid method for estimating the bacterial load of pathogens on contaminated areas.

1.2 Limitations of the study

- Multiplex qPCR is generally costly. Therefore, *Salmonella* spp. and *Campylobacter* spp. were assayed by qPCR while *S. aureus* and *E. coli* were enumerated by 3M Petrifilm™.
- Both live and dead cells can be detected when quantifying pathogens by qPCR analysis.

2 Literature Review

2.1 Introduction

Disease surveillance is a monitoring program for the occurrence of diseases in a population. It is defined as "an ongoing systematic collection, collation, analysis, and interpretation of health data, that is essential to planning, implementation, and evaluation of the public health practice" (WHO, 2000). EpiSurv is New Zealand's disease surveillance system under the Health Act 1956, which records notified diseases around the country (MPI, 2014).

In New Zealand, the reported outbreaks and cases in 2015, decreased to 558 outbreaks (♬ 35 %) and 8,510 cases (♬ 42.6 %), compared with 2014 (863 outbreaks involving 14,825 cases) (ESR, 2016). About 14% (78/558) of the outbreaks were due to foodborne diseases, and about 68 % (53/78 outbreaks) of these were linked to foodborne pathogens (ESR, 2016). Enteric bacteria caused about 32 % (25/78) of foodborne outbreaks, where *Campylobacter* was reported to be the most common (14.1 %, 11/78 outbreaks) (ESR, 2016). Of the 78 foodborne outbreaks, only 23 % (18/78) identified the sources of infection (ESR, 2016). Identifying the main source of infection is generally difficult because some people with infections may not seek medical attention, or diagnosed due to the non-specific nature of the symptoms (ESR, 2016; Ford, Miller, Cawthorne, Fearnley, & Kirk, 2015). The main foods implicated were poultry (17 %, 5 outbreaks), dairy and sugars (22 %, 4 outbreaks each), followed by grains/beans (17 %, 3 outbreaks) (ESR, 2016).

Throughout the years, the public demand for poultry has continuously increased due to their affordability and health benefits (source of protein, vitamins, and minerals) (Pattison, 2008). Poultry is now produced in broiler farms, processed industrially and sold in supermarkets. However, because of public demands, this type of food production gives an opportunity for foodborne pathogens and toxins to infect large numbers of consumers (Sanders, 1999).

Implementing mitigation strategies in broiler production controls pathogen contamination in poultry products, and decreases foodborne outbreaks. However, to implement successful control strategies, understanding pathogen prevalence and transmission routes are important. This literature review will be focusing on four pathogens, namely *Campylobacter* spp., *Salmonella* spp., *E. coli*, and *S. aureus.*

2.2 General characteristics of *Campylobacter* spp.

Most reported outbreaks of bacterial gastroenteritis have been associated with *Campylobacter* spp. (ESR, 2016). *Campylobacter* spp. are Gram -ve, curved/ spiral rods, non-spore forming and motile bacteria (Leedom Larson & Spickler, 2013). The motility of *Campylobacter* spp. is important for colonisation and infection of various food products. They were observed to have a rapid darting action due to their long sheathed polar (one) or bipolar (two) flagellum (FDA, 2013).

Thermophilic *Campylobacter* spp. are often isolated in foodborne outbreaks (Ugarte-Ruiz et al., 2012). *Campylobacter* spp. rapidly grows in temperatures between 35 and 42 ºC, but are incapable of surviving bellow 30 ºC (Silva et al., 2011). Their inability to adapt and grow at lower temperatures are explained by the absence of a cold shock protein gene, that most bacteria have (Levin, 2007). Thermophilic *Campylobacter* spp. uses amino acids and intermediates from the citric acid cycle as their energy source, instead of oxidising carbohydrates. They are microaerophilic organisms, that requires oxygen levels between two to 10 % for growth (Silva et al., 2011).

There are a variety of biochemical tests available for *Campylobacter* spp. identification. The most common are catalase, oxidase, nitrate reduction, and hippurate hydrolysis (Silva et al., 2011). Table 2.1 illustrates the characteristics of each *Campylobacter* isolates for species differentiation, specifically for *C. fetus, C. jejuni, C. coli, C. sputorum*, and *C. concisus*

Table 2.1 Characteristics *Campylobacter* spp. for species differentiation

	Cb. fetus[a]	Cb. jejuni	Cb. coli	Cb. sputorum[b]	Cb. concisus
Genus *Campylobacter*					
Motility	+	+	+	+	+
Oxidation/fermentation	–/–	–/–	–/–	–/–	–/–
Oxidase	+	+	+	+	+
NO_3 reduction	+	+	+	+	+
Methyl red	–	–	–	–	–
Voges Proskauer	–	–	–	–	–
Indole	–	–	–	–	–
Lipase	–	–	–	–	–
Gelatinase	–	–	–	–	–
Urease	–	–	–	–	–
Catalase	+	+	+	–	–
NO_2 reduction	–	–	–	+	+
H_2S production[c]	–	–	–	+	+
H_2S production[d]	v	+	+	+	+
Hippurate hydrolysis	–	+	–	–	?
Indoxylacetate hydrolysis (IAH test)	–	+	–	–	–
Growth in/at glycine 1%	v	+	+	v	–
NaCl 3.5%	–	–	–	v	–
25 °C	+	–	–	(–)	–
42 °C	–	+	+	v	–
Resistance against nalidixic acid	+	–	–	v	+
Cephalotin	–	+	+	–	?

[a] Including subspecies.
[b] Including subspecies.
[c] TSI/SIM , triplesugariron/SIMmedium.
[d] pH-acetate strip
Source: Teufel (2002)

2.2.1 Viable *Campylobacter* but non-culturable state

Environmental microbiologists were the first to describe viable, but non-culturable physiological state of *Campylobacter* spp. (Tholozan, Cappelier, Tissier, Delattre, & Federighi, 1999). For instance, *Campylobacter* spp. changes their morphology into a non-culturable state, when exposed to adverse environmental conditions, such as temperature and atmospheric stress (Silva et al., 2011). Despite their non-culturable state, they are not regarded as dead cells because their cell membrane is still intact, and their genetic information are not damaged (Li, Mendis, Trigui, Oliver, & Faucher, 2014). It was suggested that the viable but non-culturable physiological state of *Campylobacter* spp. may have a role in most food poisoning incidents (Tholozan et al., 1999).

2.2.2 *Campylobacter* and human illness

Human campylobacteriosis is increasing around the world due to the consumption of contaminated food and water. It has been reported that the most prevalent source of campylobacteriosis is from undercooked poultry meat, and the mishandling of raw poultry (Heyndrickx et al., 2002; Teufel, 2002)

C. jejuni and *C. coli* causes gastroenteritis. Depending on the dosage of the bacterium, the disease can last for 1 to 10 days (MPI, 2001), and cause acute enteritis (abdominal cramps, fever, headache, severe diarrhoea and muscle pain)**,** or acute inflammatory enterocolitis (inflammation of the inner lining of the colon, resulting in bloody stools) (FDA, 2013; MPI, 2001).

Campylobacter spp. infections result in severe complications when treated incorrectly. Complications such as bacteraemia (presence of bacteria in blood), pancreatitis (infection of liver and pancreas), miscarriage and hepatitis have been reported worldwide (WHO, 2009). Autoimmune disorders, such as Guillain-Barre syndrome (GBS), were also observed to be associated with campylobacteriosis (FDA, 2013). GBS is a type of paralysis that results in respiratory and severe neurological dysfunction. Antigens present in *C. jejuni*, are similar to those found in the nervous tissues of humans, which explains the autoimmune reaction of GBS (MPI, 2014).

2.3 General characteristics of *Salmonella* spp.

Salmonella spp. are Gram -ve, rod-shaped, facultative bacterium (Singh, 2013). All *Salmonella* spp. are flagellated, except for *S.* Gallinarum and *S.* Pullorum (FDA, 2013). *Salmonella* can survive in temperatures between 7 to 54 ºC, with an optimal temperature of 37 ºC. They can also survive in an environment that has a pH range between 4 to 9.5 (Singh, 2013).

Biochemical tests have been used for differentiating bacterial species (Table 2.2). *Salmonella* spp. were observed to reduce nitrates to nitrites, produce hydrogen sulphide,

and ferment D-glucose to produce carbon dioxide and hydrogen gas (except for *S.* Typhi) (Cosby et al., 2015).

Table 2.2 *Salmonella* spp. characteristics for species differentiation

Characteristics	*Salmonella enterica* subsp.						*Salmonella bongori*
	enterica	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Classification (roman numeral)	I	II	IIIa	IIIb	IV	VI	V (formerly)
Usual habitat	Warm-blooded animals	Warm-blooded animals	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment
Morphological characteristics							
Gram stain	-	-	-	-	-	-	-
Motility	+ (except pullorum & gallinarum)	+	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Size (width, µm)	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5	0.7-1.5
Size (length, µm)	2-5	2-5	2-5	2-5	2-5	2-5	2-5
Colony morphologies							
Bismuth sulphite agar	Black colonies surrounded by a brown to black zone that casts a metallic sheen						
Eosin-methylene blue agar	Translucent amber to colourless colonies						
Hektoen enteric agar	Blue to blue-green colonies, mostly with black centers (H_2S producers)						
Salmonella-Shigella agar	Colourless colonies on a pink background						
Xylose lysine desoxycholate agar	Black-centered red colonies (H_2S producers)						
Growth characteristics							
Optimum temperature (°C)	35-37	35-37	35-37	35-37	35-37	35-37	35-37
Optimum pH	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5
Biochemical characteristics							
α-glutamyltransferase	d	+	-	+	+	+	+
β-Gluocuronidase	d	d	-	+	-	d	-
Dulcitol	+	+	-	-	-	d	+
Galacturonate	-	+	-	+	+	+	+
Gelatinase	-	+	+	+	+	+	-
Glucose	+	+	+	+	+	+	+
Hydrogen sulfide	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-
Lactose	-	-	-	+	-	+	d
Lysine decarboxylase	+	+	+	+	+	+	+
L(+)-tartrate	+	-	-	-	-	-	-
Malonate	-	+	+	+	-	-	-
Methyl red test	+	+	+	+	+	+	+
Murate	+	+	+	-	-	+	+
Ortho-nitrophenyl-β-D-Galactopyranoside test	-	-	+	+	-	d	+
Phage O1 susceptible	+	+	-	+	-	+	d
Potassium cyanide broth	-	-	-	-	-	-	-
Salicine	-	-	-	+	-	+	d
Sorbitol	+	+	+	+	+	-	+
Urease	-	-	-	-	-	-	-
Voger-Proskauer test	-	-	-	-	-	-	-

+, more than 90% positive reactions; -, less than 10% positive reactions; d, different reactions given by different serovars.

Source: Pui et al. (2011)

2.3.1 *Salmonella* and human illness

Salmonellosis has been an economic and health problem among non-industrial and industrial countries (Pui et al., 2011). *Salmonella* spp. were observed to cause infection

through the consumption of contaminated food (meat, chicken, milk, eggs) and water (Forshell & Wierup, 2006; Singh, 2013). Once ingested, *Salmonella* multiplies in the small intestine, enabling them to invade intestinal walls, and spread to other parts of the body (Singh, 2013).

Salmonella causes two types of illnesses that are dependent on the serotype involved (FDA, 2013). *S*. Typhi and *S.* Paratyphi are known to cause typhoid fever, whilst other *Salmonella* spp. causes non-typhoidal salmonellosis (Forshell & Wierup, 2006). Salmonellosis is a type of acute enteritis that causes diarrhoea, nausea, mild fever, and chills, after 12 hours of infection (FDA, 2013). Salmonellosis typically lasts for one week, but it entirely depends on the dosage of the pathogen, as well as peoples' susceptibility to the disease (Forshell & Wierup, 2006). For instance, young infants and children that are immune deficient are most at risk, because their immune system is incapable of protecting them against foreign invaders (AAAAI, 2015).

2.4 General characteristics of *Escherichia coli*

Escherichia coli (*E. coli)* are Gram -ve, motile, flagellated (150 μm long), medium sized (2 to 3 μm long) rods, that are normally found on the intestines of warm-blooded animals (La Ragione & Woodward, 2002; Winfield & Groisman, 2003). Most strains are observed to be non-invasive, and provide protection against disease causing bacteria (Odonkor & Ampofo, 2013). However, some are known to cause severe infections and foodborne diseases (Caprioli et al., 2005).

E. coli can survive and tolerate environmental stressors. Their ideal temperature for growth is 7 to 46 ˚C, with an optimal temperature of 37 ˚C. They are also able to survive in environments with a pH range between 4.4 to 9 (Shaw, Lake, & Whyte, 2003). *E. coli* are observed to survive in chilled foods, as well as low pH environments (Park et al., 2009).

2.4.1 Nomenclature of *Escherichia coli*

E. coli strains are characterised based on the presence of their somatic (O) and flagellar (H) antigens (Shaw et al., 2003). However, pathogenic nomenclature of *E. coli* is based on how each strain infects and cause symptoms. In recent years, diarrhoeagenic strains have been labelled enterotoxigenic *E. coli* (ETEC), if they produce toxins that does not damage host epithelium; enteroinvasive *E. coli* (EIEC), if they penetrate into host epithelial cells; enteropathogenic *E. coli* (EPEC), if they adhere to epithelial cells and cause infection; and enterohemorrhagic *E. coli* (EHEC), if they cause bloody diarrhoea like *E. coli* O157:H7 (Kaper, Nataro, & Mobley, 2004; Tarr, 1995).

2.4.2 Virulence Factors of *Escherichia coli*

Shinga toxin (Stx) producing *E. coli* (STEC) or verotoxin (VT) producing *E. coli* (VTEC), are characterised by their production of cytotoxins (Beutin, 2006; Caprioli et al., 2005). These toxins are known as Stx because of their similarity with *Shigella dysenteriae* toxins, or VT because of their activity on vero cells in tissue culture (Caprioli et al., 2005; Shaw et al., 2003).

Stx1 (VT1) and Stx2 (VT2) genes are responsible for the Stx production (Beutin, 2006; Kaper et al., 2004). The genes are found in the genome of temperate bacteriophages, which integrate into the STEC chromosome (Beutin, 2006). Epidemiological studies reveal that Stx2 was frequently associated with foodborne diseases, compared to Stx1 (Caprioli et al., 2005). However, the severity of the disease depends on the variants produced by *E. coli* strains. Each variant has different antigenic and biological characteristics. For instance, Stx2 variants such as Stx2a and Stx2c, were found in strains isolated from patients with haemorrhagic colitis. While Stx2d variant producing strain were isolated from cases with mild diarrhoea (Caprioli et al., 2005).

Most STEC strains colonise intestinal mucosa of hosts and induce histopathological intestinal lesions (Kaper et al., 2004). These lesions are described as “attaching and effacing” (A/E) lesions, where the bacteria attaches to the intestinal lining of the host, and

disrupt epithelial cell functions (Caprioli et al., 2005; Jerse, Yu, Tall, & Kaper, 1990).

A 35 KB chromosomal pathogenicity island called the locus of enterocyte effacement (LEE), encodes the genes involved for A/E lesions, which are essential for pathogen invasions (Elliott et al., 1998; Kaper et al., 2004). The first set of genes encodes for type III secretion systems that exports effector molecules. The second set encodes for secreted proteins, important for disrupting cytoskeleton of the host. The third set, *eae* gene, encodes for intimin, which aids adhesion of the bacteria to the host cell wall. Whilst *tir* gene encodes for an intimin receptor protein that enables translocation through host cell membranes (Caprioli et al., 2005; Elliott et al., 1998; Mellies, Elliott, Sperandio, Donnenberg, & Kaper, 1999). A knockout study of LEE genes on *E. coli* 0157:H7, demonstrated the inability of A/E pathogens to colonise host cells and cause severe diseases (Doughty et al., 2002). However, some serotypes of STEC are LEE negative strains, and are still capable of causing human disease outbreaks (Newton et al., 2009).

Little is known about the colonisation of LEE negative strains. However, it was observed that these strains interact with the host's intestinal mucosa, and cause diseases. For instance, LEE negative strains, such as STEC 0113, consist of a genetic locus (ssa) that enables them to colonise by aggregation, onto epithelial cells (Caprioli et al., 2005). It was also observed that an STEC autotransporter protein (sab), contributes to the adherence and the colonisation of LEE negative strains by biofilm formation (Croxen et al., 2013).

2.4.3 *E. coli* and human illness

Among the pathogenic *E. coli* serotypes, *E. coli* O157:H7 was frequently observed to cause severe infections, especially in industrialised countries (Caprioli et al., 2005). *E. coli* infections occur due to the ingestion of contaminated food (raw or undercooked meat products, milk, vegetables) and water. Most *E. coli* contamination was observed to be from animal faeces contact during cultivation, or person-to-person contact through the oral-faecal route (Shaw et al., 2003).

The magnitude and severity of *E. coli* infections vary from person to person. Most strains invade the gut, and produce toxins that cause mild symptoms, such as abdominal pain and watery diarrhoea (Beutin, 2006; Peacock, Jacob, & Fallone, 2001). However, other strains also causes severe symptoms including haemorrhagic colitis (bloody diarrhoea, vomiting, abdominal pain), haemolytic-uraemic syndrome (renal failure, mostly associated with children), and thrombotic thrombocytopenic purpura (fever and nervous system disorder, which is mostly experienced by the elderly) (Beutin, 2006; Shaw et al., 2003).

2.5 General characteristics of *Staphylococcus aureus*

Staphylococcus aureus (S. aureus) is a pathogen that causes hospital and community acquired infections, because of their "invasive and antibiotic resistant" characteristics (Kadariya, Smith, & Thapaliya, 2014). *S. aureus* are Gram +ve, non-motile, spherical bacteria (cocci). They are found in pairs, short chains or bunched in grape-like clusters (FDA, 2013). *S. aureus* can grow in a wide range of temperatures (7 to 48.5 ºC; optimum 30 to 37 ºC), pH (4.2 to 9.3; optimum 7), and salt concentrations (up to 15% NaCl), which explains their ability to grow in various food products (FDA, 2013; Le Loir, Baron, & Gautier, 2003)**.**

2.5.1 *S. aureus* Enterotoxins

S. aureus produces toxins, called staphylococcal enterotoxins (SE), which are single chained proteins that are resistant to proteolytic enzymes (trypsin and pepsin) of the digestive tract (FDA, 2013). There are nine serological types of SE (SEA, SEB, SEC, SED, SEE, SEG, SHE, SEI, SEJ) that belongs to the family of super antigens (Kadariya et al., 2014). These super antigens are resistant to severe conditions (heat treatment, low pH) that easily destroys the microorganism that produces them (Argudín, Mendoza, & Rodicio, 2010).

SE causes immunosuppression due to non-specific T-cell proliferation (Kadariya et al., 2014). Normally, T-cell activation occurs when antigens bound to the major

histocompatibility complex (MHC) of antigen-presenting cells (APC), interact with T-cell antigen receptors (TcR) (Le Loir et al., 2003; Pinchuk et al., 2010). TcR is a glycosylated heterodimer that are composed of either: a and b, or d and g chains, making them recognise only specific antigens for T-cell activation, and initiate a cellular immune response (Le Loir et al., 2003). However, SE are also capable of causing T-cell activation (Figure 2.1). When SE binds to MHC of the APC, it makes a cross-link to the TcR, enabling non-specific T-cell activation (Le Loir et al., 2003). This interaction, then results in the proliferation and secretion of interleukins that are involved in SE toxicity (Le Loir et al., 2003; Pinchuk et al., 2010; Roy et al., 2002).

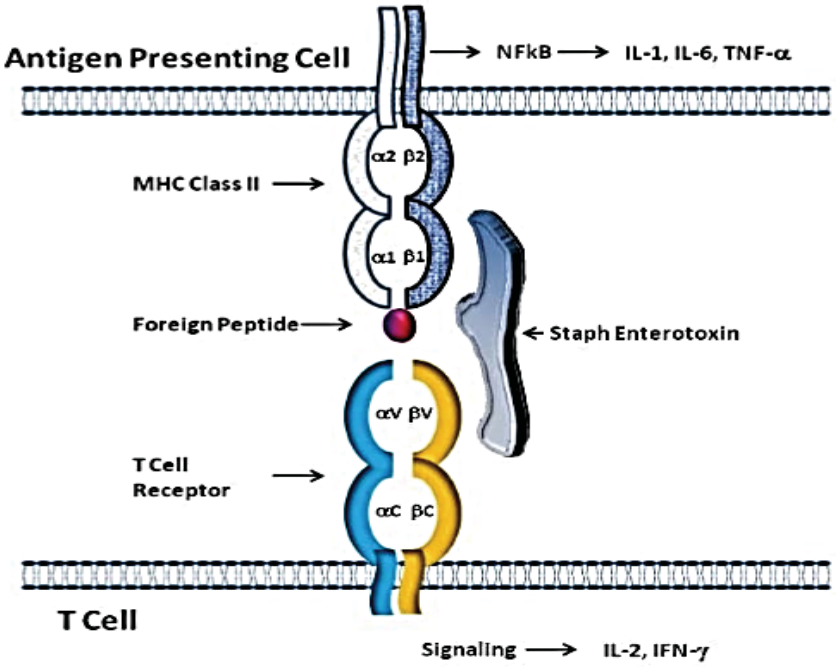

Figure 2.1 Model of non-specific SE interaction with TCR and MHC class II
Source: Pinchuk, Beswick, & Reyes (2010)

2.5.2 *S. aureus* and Human Illness

SE causes foodborne diseases around the world, due to the mishandling of processed foods (Le Loir et al., 2003). In 1985, milk chocolate caused *Staphylococcus* food poisoning in the United States. It was determined that the milk was not properly stored at

high temperatures before milk pasteurisation. The pasteurisation stage killed off most pathogens that were present, but failed to kill off SE (Le Loir et al., 2003).

Enterotoxins that causes gastroenteritis manifests quickly. Their symptoms include hyper-salivation, nausea, vomiting, and diarrhoea (Kadariya et al., 2014). Symptoms usually settle within one or two days of infection, depending on the dosage of the toxin and the age of the infected individual (FDA, 2013). However, in highly sensitive people, the ingestion of 100 ng of enterotoxin can cause *Staphylococcus* foodborne infection (FDA, 2013).

2.5.3 Methicillin resistant *S. aureus*

The excessive use of antibiotics to control *S. aureus* food poisoning incidents, caused methicillin resistant strains to emerge. Methicillin is an antibiotic that was used to treat *S. aureus* infections (NIAID, 2008). However, in 1961, British scientists discovered methicillin resistant *S. aureus* (MRSA) strains (NIAID, 2008). MRSA acquires the mecA gene that codes for penicillin binding proteins (PCP2a), and interferes with the effects of beta-lactam antibiotics. Through the years, MRSA has become increasingly resistant to all beta-lactam antibiotics (penicillin, amoxicillin, oxacillin, and methicillin), making them difficult to control (Sahare, Moon, & Shinde, 2013; Stapleton & Taylor, 2002).

2.6 Vertical transmission of pathogens in poultry

The term 'vertical transmission' firstly emerged in the 1940s to cover four contingencies, such as transplacental, transmammary, transovarial, and transovum. It was defined as 'the direct transfer of infection from a parent organism to its progeny' (Fine, 1975). Nevertheless, maternal antibiotics can be inherited, allowing low prevalence of pathogen infections (Sahin, Morishita, & Zhang, 2002). The avian gut undergoes physiological changes during the first few weeks of growth, making them more resistant to infections (Bull et al., 2006; Newell & Fearnley, 2003). However, maternal resistance declines when chicks reach 14 days of age, due to environmental factors (Newell & Fearnley, 2003).

2.6.1 Vertical transmission of *Campylobacter* spp.

Vertical transmission of *Campylobacter* spp. to flocks via contaminated eggs is controversial, due to its rare occurrence (WHO, 2009). *Campylobacter* spp. persists in various locations of the birds' reproductive tract (Newell & Fearnley, 2003). Genotyping studies revealed that *Campylobacter* strains that thrive in the reproductive tract of birds, were identical to those found in faeces (Baker et al., 2002; Newell & Fearnley, 2003; Sahin et al., 2002). As the egg passes through the birds' cloaca, faeces contaminate the external surface of the egg, enabling *Campylobacter* spp. to penetrate through the eggshell cracks (Baker et al., 2002; Newell & Fearnley, 2003). Once *Campylobacter* spp. penetrates into the egg contents, they were able to survive for up to two weeks, enabling them to infect bird hatchlings (Newell & Fearnley, 2003; Sahin et al., 2002).

2.6.2 Vertical transmission of *Salmonella* spp.

S. Enteritidis were observed to cause egg contamination, and cause major foodborne illnesses (Cox et al., 2000). It was previously thought that the main transmission route was through bacterial penetration of eggshell cracks (Gantois et al., 2009). However, because of *S.* Enteritidis' ability to surpass the birds' host defence mechanism, infection was most likely to occur in the reproductive tract, before egg shell formation (Cox et al., 2000; Foley et al., 2011; Gantois et al., 2009).

This phenomenon was investigated by inoculating *S.* Enteritidis into various parts of the bird. Bacterial inoculation through the veins caused colonisation of the ovary, as well as contamination of forming eggs in the oviduct (Gantois et al., 2009). This suggests that bird ingesting contaminated feed or water could cause food poisoning incidents. Moreover, inoculation through the vagina led to the colonisation of the lower oviduct, resulting in internally contaminated eggs (Cox et al., 2000; Gantois et al., 2009). As the egg passes through the colonised vagina, the pathogen could then penetrate through the eggshell caused by a negative pressure from egg cooling (Cox et al., 2000).

2.6.3 Vertical transmission of *E. coli*

E. coli thrives in the lower intestine of a host as it provides vast supply of nutrients and minerals for growth (Winfield & Groisman, 2003). They are considered as secondary or opportunistic pathogens because they are often isolated from the intestinal tract of healthy birds (Porter, 1998). Caprioli et al. (2005) suggests that the timing of infection reflects the onset of the disease. As soon as *E. coli* penetrates through the egg barriers and into the yolk sac, they become pathogenic after hatching because of the presence of virulent plasmids.

2.6.4 Vertical transmission of *S. aureus*

There is no evidence that *S. aureus* infects through vertical transmission (Persoons et al., 2009). *S. aureus* infections were observed to be low during the first few weeks of the chicks' life, but tends to increase when they get older (Wendlandt et al., 2013). This suggests MRSA infections are only horizontally transmitted in poultry.

2.7 Horizontal transmission of pathogens in poultry

Horizontal transmission is the most likely cause of pathogen outbreaks in broiler farms (Silva et al., 2011). Internal contamination of the farms, were due to the residual presence of pathogens from pervious infected flocks (Newell & Fearnley, 2003). Whilst external contamination of the farm, was associated to pathogens transported into broiler houses in equipment/ utilities (such as feed, litter and water), by human activities (farm workers/staff), as well as birds and insects (Newell & Fearnley, 2003; Sahin et al., 2002).

Potential risk factors associated with pathogen contamination, is dependent on management practices involved in individual broiler farms (Newell & Fearnley, 2003; Vieira, Hofacre, Smith, & Cole, 2009). These risk factors include; poor house maintenance (large rodent population, dust), poor hygiene barriers (staff hygiene and the use of boot dips), insufficient cleaning and disinfection between flocks, short empty

periods, and contaminated feed and water supplies (poorly cleaned feed trays and water pipes) (Newell & Fearnley, 2003; Vandeplas et al., 2010). With these risk factors, proper identification of sources that can cause pathogen infection to broiler birds are essential to ensure effective preventive measures (Newell & Fearnley, 2003).

2.7.1 Horizontal transmission of *Campylobacter* spp.

Studies have focused on the epidemiology of *Campylobacter*, and its potential biotic and abiotic sources (Baker et al., 2002; Guerin et al., 2007; Heyndrickx et al., 2002; Vandeplas et al., 2010). Herman et al. (2003) found no *Campylobacter* spp. contamination from samples collected on hatcheries, and one-day old chicks. This indicates that the chicks transferred to broiler farms were not infected, eliminating vertical transmission as the source of infection (Herman et al., 2003). Farms with *Campylobacter* spp. contamination manifests due to insufficient disinfection of the sheds. Faecal contamination around the sheds were also an issue because dust, pests, flies, beetles, and farm workers, can spread the pathogen (Baker et al., 2002; Herman et al., 2003)

2.7.2 Horizontal transmission of *Salmonella* spp.

Several isk factors for horizontal transmission have been identified by various studies (Alali, Thakur, Berghaus, Martin, & Gebreyes, 2010; Andino & Hanning, 2015; Heyndrickx et al., 2002; Liljebjelke et al., 2005). High contamination levels on feed trays and water drinkers were observed due to faecal droppings of *Salmonella* spp., which can then horizontally spread to other birds within the same house (Alali et al., 2010) Recontamination of broiler sheds from inefficient cleaning is also an issue, especially when pest, flies, beetles and dust, can amplify existing contamination by reaching inaccessible areas (Davies & Breslin, 2003; Heyndrickx et al., 2002; Thaker, Brahmbhatt, Nayak, & Thaker, 2013).

2.7.3 Horizontal transmission of *E. coli*

E. coli thrives in the lower intestine of a warm-blooded animal, as it provides a vast supply of nutrients for bacterial growth. However, when exposed to external environmental conditions (low nutrient availability and temperature fluctuations), *E. coli* populations may decrease by two folds (Winfield & Groisman, 2003). *E. coli* populations maintain their numbers externally by constant contamination of micro-organisms from host excretions (Winfield & Groisman, 2003). This demonstrates that *E. coli* are incapable of surviving without a host, due to the lack of nutrients and harsh environmental conditions (Beutin, 2006; Winfield & Groisman, 2003).

Nonetheless, non-host environments can effectively mimic favourable conditions of a host environment, especially during the summer months (Winfield & Groisman, 2003). For instance, faecal shedding contaminates soil and drinking water in broiler houses, which often results to the proliferation of *E. coli* growth. Moreover, pests and insects have also been found to serve as possible vectors of STEC transmissions among poultry animals (Beutin, 2006; Doyle & Erickson, 2006).

2.7.4 Horizontal transmission of *S. aureus*

Environmental transmission of MRSA isolates is difficult to determine because of its low prevalence (FSANZ, 2015). However, *S. aureus* were isolated in bruised tissues, arthritic joints, feet, and skin surfaces of broiler chickens (Roberts, Tompkin, & Baird-Parker, 1996). It was also identified that contamination of *S. aureus* in broiler farms originated in poultry faeces, contaminating feed and water sources, or are transmitted from people and insects upon entering the sheds (FSANZ, 2015; Wendlandt et al., 2013).

2.8 Commercial poultry farming

Poultry production is a growing industry that became significant in the 1940s (Wabeck, 2002). At that time, poultry meat only becomes available when unwanted chickens are killed off, because of their decreased egg production (Mench, James, Pajor, & Thompson, 2008; Wabeck, 2002). However, major developments of poultry production increased in the late 19th and early 20th century, when artificial incubators were invented (Mench et al., 2008). Soon after this, poultry nutritional requirements improved, and synthesising dietary ingredients required for effective growth were formulated, to eliminate the need to forage on pasture grounds (Broom & Fraser, 2015; Mench et al., 2008). For instance, by adding vitamin D3 in a birds' diet, birds do not require sunlight to synthesise it. These types of developments and discoveries allow year-round poultry production to occur indoors, especially because environmental conditions can be controlled for effective broiler growth (Appleby, Mench, & Hughes, 2004; Mench et al., 2008).

Genetic selection also contributes to the success of commercial poultry production (Mench et al., 2008). In the past two centuries, more than 300 pure breeds of chickens were developed to improve productivity, and meet market demand for 'meatier' chickens (Mench et al., 2008; Wabeck, 2002). Selection pressure by breeders led them to develop broiler chickens with rapid growth traits (Mench et al., 2008; Wabeck, 2002). Poultry breeding is now a large industry, where companies worldwide, maintains broiler grandparent stock, and supply parent stock for poultry production (Broom & Fraser, 2015; Mench et al., 2008).

2.8.1 Chicks transported from hatcheries to farms

Poultry industries of New Zealand use Ross bird for chicken meat production. Most chicks are transported to farm sheds from hatcheries, by using ventilated boxes (ACMF, 2013). During transportation, it is critical that the chick is sustained by providing warmth, feed and water until they reach the broiler farms (PIANZ, 2014).

2.8.2 General description of shed

Broiler farms consist of multiple sheds that accommodates more than 40,000 broiler chickens (PIANZ, 2014)**.** These sheds can vary in sizes, but are mostly 150 meters long and 15 meters wide. It contains a ventilation and heating systems that maintain desired temperatures, as well as control relative humidity (PIANZ, 2014).

Target temperatures in the sheds gradually decreases for best broiler meat production, from 30 ˚C when they arrive in broiler farms, to 20 ˚C at harvest time (Aviagen, 2009). Thermal stress is a significant problem in breeder flocks (Lara & Rostagno, 2013; Mench et al., 2008). When temperatures are not controlled and becomes too low, birds increase their feed intake to make their bodies warmer. However, if temperatures are too high, they reduce their feed intake to decrease body heat production (Appleby et al., 2004; Aviagen, 2009).

The relative temperature of the air controls the sheds humidity. Warm air absorbs moisture from birds and litter to avoid ammonia saturation (Aviagen, 2009; Mench et al., 2008). A ventilation system, then aids the humidity out of the shed, eliminating stress, making the birds more resistant to diseases (Aviagen, 2009; Wabeck, 2002)**.**

There are two types of ventilation systems in New Zealand, cross ventilation and tunnel ventilation. Cross ventilation is a common system in sheds, where fans are used to draw air into the sheds. These fans create a negative pressure inside the shed, that eventually draws the air in, through the vents (Aviagen, 2009; PIANZ, 2014). Tunnel ventilation systems, however, have fans on one end of the shed where it draws air in, towards the cooling pads on the walls, and out again through another set of fans, on the other end of the shed (PIANZ, 2014). This system produces a ‘wind-chill’ cooling effect that keeps the birds cool in warm and hot temperatures (Aviagen, 2009). Controlling environmental cues in broiler sheds are critical for chicken meat production (Calvet, Estelles, Cambra-Lopez, Torres, & Van den Weghe, 2011). The right temperature must be provided for effective growth, as well as maintaining moisture to prevent potential health problems from ammonia accumulation in litter (Appleby et al., 2004; Aviagen, 2009; Broom & Fraser, 2015; Mench et al., 2008).

Feed and water lines runs the length of the shed so that chickens are never more than two meters away from food and water. Silos from the outside of the shed automatically supplies feeds, whilst water is supplied in drinkers at regular intervals (PIANZ, 2014). New Zealand feed mostly consists of wheat, with small amounts of maize, barley, soya bean, bran, tallow, and trace amounts of lysine, methionine, salt, vitamins, and trace minerals (PIANZ, 2014).

2.8.3 Growth requirements of chicks

There are general requirements before placing chicks inside the broiler sheds. The shed must be cleaned and disinfected, with fresh litter; as well as having the shed pre-heated or cooled at the desired temperature (ACMF, 2013).

On arrival of the day old chicks, they undergo brooding in a confined area of the shed, with food, water, and heaters (brooders) set up at temperatures between 31 – 32 ˚C (ACMF, 2013; Appleby et al., 2004). As the chicks grow, the temperature gradually decreases by 0.5 ˚C each day, until it reaches 21 – 23 ˚C (ACMF, 2013). Brooders eventually gets removed depending on the climate of the shed, and more space becomes available as the chicks grow. Farmers constantly check the sheds humidity, temperature, feeders and waters, as well as the health and performance of the birds (ACMF, 2013; Appleby et al., 2004).

2.8.4 Harvesting grown chickens

Chickens are aimed to be harvested at night, because birds are more settled and stress free in cooler temperatures (ACMF, 2013; Hui & Guerrero-Legarreta, 2010). Chickens are caught and placed into plastic crates designed to safely transport them to the processing plants (ACMF, 2013).

2.8.5 Cleaning and disinfection of sheds

When all birds were harvested, and transported to processing plants, the sheds are cleaned, disinfected and prepared for the next batch of day old chicks (ACMF, 2013). Cleaning and disinfection regimes usually occur for a week, to reduce the risk of pathogen infection being passed on between batches (ACMF, 2013).

Cleaning procedures include removing wet shavings (litter) and feed pellets out of the sheds; blowing out surface dust from ceilings, fans, water pipes, and loose debris on floors; as well as disinfecting the drinking water system (ACMF, 2013; Pattison, 2008). Disinfecting the water system avoids infection, but this procedure is often ignored. Cleaning the water system involves draining the head tank empty, filling the head tank with diluted disinfectant, and leaving the solution to saturate for at least an hour. After an hour, the head tank is flushed and drained thoroughly. It is then filled up with fresh water, and covered to reduce recontamination (On, Lake, & Wong, 2008; Pattison, 2008).

A pre-disinfectant solution is applied to all surfaces of the shed by using a high-pressure water blaster (Gietema, 2002; Ray & Bhunia, 2007). The pre-disinfectant solutions efficiency is dependent on how well it emulsifies lipids, dissolve proteins, and solubilize or suspend carbohydrates and minerals (Ray & Bhunia, 2007). Detergents are either cationic, anionic, or non-ionic. Anionic detergents are preferred when disinfecting the sheds due to its polar (hydrophilic or lipophobic) and nonpolar (hydrophobic or lipophilic) segments. Their hydrophobic segment for instance, helps dissolve lipid materials in soil, by forming micelles (Ray & Bhunia, 2007; Sanchez-Ferrer, Bru, & Garcia-Carmona, 1994). This pre-disinfection procedure aims to remove all organic matter and biofilms on surfaces, to increase the efficacy of the main disinfectant used (ACMF, 2013).

Disinfectants must be thoroughly applied on all surfaces and equipment of the shed (Gietema, 2002). It is used to effectively destroy pathogenic microorganisms, and reduce microbial loads. They eliminate all microorganisms by membrane disruption, metabolic inhibition, and cell lysis (Soliman, Sobeih, Ahmad, Hussein, & Moneim, 2009). Important factors for the antimicrobial efficiency of the disinfectant is exposure, time,

temperature, concentration used, pH, microbial attachment to surface, microbial load, and water hardness (Ray & Bhunia, 2007).

2.9 Pathogen control measures in poultry production

Pathogen control measures in poultry involve operational procedures that ensure control during production. The following points below are some of the critical control points that must be considered in a biosecurity program of poultry farming.

2.9.1 Footbaths in the annex

Footbaths were observed to contribute to spreading contamination in the main shed, especially when it is not properly used (only dipping toes or heels, passing through the disinfectant very quickly, and low frequency of changing the dip) (McDowell et al., 2008; Robyn, Rasschaert, Pasmans, & Heyndrickx, 2015). It was recommended by Evans and Sayers (2000) that changing disinfectant solutions in footbaths at least once a week, and certainly when there has been a build-up of organic matter, reduces flock infection. Allen and Newell (2005) also suggests that the use of dedicated boots and using footbaths before entering the main shed, increases protection against contamination.

2.9.2 Feed treatments

Feed are potential vectors for several pathogens, such as *Salmonella* spp., *Clostridium* spp. and *E. coli*. Contamination can occur in raw materials, during production, during storage or in transport vehicles (Pattison, 2008).

Feed producers use a variety of treatments to reduce pathogen load in the feed (Doyle & Erickson, 2006) Organic acids were used with feed as an additive, to prevent food deterioration and control for microbial contamination. Organic acids are non-corrosive

and do not cause harm to the animals' growth or health (Ricke, 2003). Moreover, cooking and pelleting feed at high temperatures (between 70 °C – 90 °C) were carried out to eliminate pathogen growth. However, the efficacy of heat treatment is entirely dependent on the moisture content of the feed (Doyle & Erickson, 2006). For instance, heat treatment at 82 °C was used on feed that contained 15 % moisture, will have a bacterial Log reduction of 4.5; Whilst feed containing 5 % moisture will only have 1.5 Log reduction (Doyle & Erickson, 2006). Combining both treatments during feed production will be more effective at eliminating pathogens, than using chemical or heat treatments separately (Doyle & Erickson, 2006).

2.9.3 Water treatments

Pathogenic bacteria frequently contaminate water systems in poultry farms. Bacteria on feed particles, dust, litter, and faeces contaminates open drinkers (bell drinkers), thus infecting poultry flocks (Appleby et al., 2004; Pattison, 2008). New flocks, in particular, are easily infected due to their low bacterial resistance (Sadler, Brownell, & Fanelli, 1969). They are susceptible to diseases when exposed to the contaminated environment, feed or water (Erf, 1997). Therefore, closed water systems (nipple drinkers) are preferred by poultry farmers, as it reduces the chance of pathogen contamination in water. However, pathogen infections are still possible in closed water systems, especially when residual contamination is present. Efficient disinfection must still be applied in water tanks, and water systems, to effectively eliminate possible flock infections (Manning, Chadd, & Baines, 2007; Pattison, 2008).

Chemical treatments are applied on water sources to control for pathogen contamination (Doyle & Erickson, 2006). Adding diluted organic acids in drinking water was favoured as a biosecurity strategy because it does not damage the epithelial cells of the digestive tract of birds when consumed (Chaveerach, Keuzenkamp, Lipman, & Van Knapen, 2004). Moreover, diluted organic acids also eliminate the growth of biofilms. Biofilms provide nutrients and oxygen to water-borne pathogens. Applying chemical treatments in water sources hinders biofilm growth, which then controls waterborne infections (Manning et al., 2007; Pattison, 2008)

2.9.4 Pests

Poultry sheds attract rats due to its warmth, shelter, and food availability. Rats are known to carry several pathogens, where *S.* Enteritidis and *S.* Typhimurium are the most common. An effective rodent control program must be implemented and maintained. The program should include monitoring rodent activity (possible damage to the building), monitoring the presence of droppings, as well as monitoring baits (Pattison, 2008).

Darkling beetles (*Alphitobius diaperinus*) are also known as vectors for a variety of pathogens, including *Campylobacter, E. coli, Aspergillus* and *Salmonella*. Monitoring darkling beetles are of great importance not only because they are vectors of pathogens, but also because they have the ability to cause damage in poultry shed insulation (Bates, Hiett, & Stern, 2004). In addition, flies were implicated as seasonal vectors of *Campylobacter* spp. (Pattison, 2008). It was observed by Bahrndorff, Rangstrup-Christensen, Nordentoft, and Hald (2013) that the prevalence of *Campylobacter* spp. infection peaks during the summer months due to the abundance of flies (Bahrndorff et al., 2013).

2.9.5 Dry Litter

Litter is used in poultry houses, mainly to control for ammonia accumulation. Ammonia in broiler houses reduces growth rate, as well as increase potential pathogen outbreaks (Manning et al., 2007; Wabeck, 2002). Litter is known to aid climate control in broiler houses, thus minimising pathogen infection of birds. Litter absorbs moisture when broiler conditions are damp, but then they release this absorbance during the dry periods (Wabeck, 2002). Nonetheless, problems can arise during rearing and causes wet litter. Table 2.3 lists the causes and consequences of wet litter when they are not properly managed.

Table 2.3 Causes and consequences of wet litter during rearing

Causes	Consequences
• Restriction of feed, increases water consumption, which results to diarrhoea • Excessive sodium, potassium and magnesium intake from feed increases water intake and excretion • Excess protein intake increases water intake and allows the excretion of high uric acid levels, and loose droppings • Excess levels of sugars leads to osmotic changes that leads to lower bowel fermentation and loose droppings • Bacteria contamination in feed and water can cause excessive diarrhoea	• Excessive ammonia in the house due to fermentation of urea (urate in faeces) by urate-splitting bacteria in warm, moist conditions • Ammonia reduces appetite and damages the respiratory system • Coccidiosis infection • Soiling of birds, breast blisters and loose droppings in broilers have welfare implications

Source: Pattison (2008)

2.9.6 Site decontamination

In the past, a range of chemicals was used as disinfectants in broiler sheds. These chemicals were observed to achieve low efficacy, due to its correlation with inefficient method of application (Pattison, 2008). But also, causes harm to the environment due to its toxic and corrosive nature. To date, newly developed disinfectants offers high efficacy, and less environmental implications (Pattison, 2008). Selecting the right type of disinfectant, depends on various factors. This includes the type of surface being disinfected, the level of organic material present, temperature, water quality and contact time (Pattison, 2008). Table 2.4 gives a brief overview of each factor.

Table 2.4 Factors affecting disinfection efficiency

Type of surface	Some poultry houses are constructed with materials that has rough surfaces. The ability of the disinfectant to penetrate these types of surfaces are significant.
Water quality	Water hardness has a significant effect on the activity of some disinfectant. For instance, phenolic compounds are less affected then idophores.
Contact time	Contact time varies with different disinfectants, to achieve maximum results. For example, oxidising systems are fast acting, but are slower.
Organic materials	Organic materials have a negative effect on all chemical disinfectants. For effective hygiene control, cleaning and washing of surfaces is a requirement before applying disinfectants. Some surfaces are easier to clean than others, and there are also different amounts of organic residues that needs to be considered.
Temperature	The activity of disinfectants usually increases with temperature. However, this varies greatly with the chemical composition of the disinfectant. Increase contact time, and lowering the dilution of the chemical may be necessary in winter.

Source: Pattison (2008)

2.10 Experimental Background

2.10.1 Determination of sample size using Win Episcope 2.0

Controlling infectious diseases among livestock has been a critical focus in both developed and undeveloped countries. Recent advances in methods and statistical theory, to support quantitative procedures for investigating disease frequencies has been described by Niskanen and Pohja (1977), and Martin, Meek, and Willeberg (1987). The statistical package, Win Episcope 2.0 (Wageningen Agricultural University; http://www.zod.wau.nl/genr/epi.html), was therefore designed to aid procedures that are used in the analysis of epidemiological studies Thrusfield, Ortega, de Blas, Noordhuizen, and Frankena (2001). Most studies have used Win Episcope 2.0 to determine sampling size and sampling frequency based on observed prevalence (Castro-Hermida, Gonzalez-Warleta, & Mezo, 2015; Esteban, Oporto, Aduriz, Juste, & Hurtado, 2009; Kich et al., 2011; Patchanee et al., 2014; Schulz et al., 2011; Vanantwerpen, Van Damme, De Zutter, & Houf, 2014). The detection of disease module of Win Episcope 2.0, determines the sample size required at a specific level of confidence and assumed the prevalence of the disease (Thrusfield et al., 2001). For instance, Patchanee et al. (2014) used Win Episcope 2.0 to design sample size with an expected prevalence of 20 % and 95 % confidence interval, to investigate the occurrence and characterisation of MRSA in pigs. Castro-Hermida et al. (2015) used Win Episcope 2.0 to design sample size with 50 % prevalence and 95 % confidence interval, to investigate pathogenic contaminated water. Whilst Kich et al. (2011) also designed sample size with 50 % prevalence and 95 % confidence interval, to investigate the distribution of *Salmonella* from swine finishing herds.

2.10.2 Environmental sampling methods

Various types of surfaces (plastic, stainless steel, glass, and wood) are being used by food industries today. These surfaces are prone to bacterial contamination and form biofilms if not properly disinfected. Therefore, a method for recovering microorganisms from various types of surfaces is necessary for investigating potential contamination, to prevent bacterial outbreaks (Ismaïl et al., 2013). There are different sampling methods used to

acquire detectable pathogens. Methods such as contact plate method, and swabbing methods are most commonly used in food industries (Niskanen & Pohja, 1977).

Contact plate method involves touching the surface being examined, with the surface of an agar to produce a 'mirrored image' (Niskanen & Pohja, 1977). This method is quite efficient as it can be incubated after sampling, without the need of an enrichment step (Ismaïl et al., 2013). However, surfaces being tested with contact agar must be flat, smooth, and clean enough to produce reliable results, as well as eliminating the chance of colonies overcrowding the (Favero, McDade, Robertsen, Hoffman, & Edwards, 1968; Ismaïl et al., 2013; Pérez-Rodríguez, Valero, Carrasco, García, & Zurera, 2008).

Swabbing method, on the other hand, involves rubbing surfaces with sterile cotton swabs that are either pre-moistened with diluents or are used dry (Ismaïl et al., 2013). Quantitative or qualitative assessment of the sampled area was then carried out by releasing bacteria from the cotton swabs, into the extracting solution by vortexing /mixing, followed by enrichment, direct plating or dilution plating. This method is reliable for quantitative or qualitative assessments because it can sample large surface areas, as well as uneven and heavily contaminated surfaces (Ismaïl et al., 2013). However, limitations exist in swabbing methods. For instance, reproducibility has been reported to be poor between samplers because of the different pressures applied on the surfaces (Pérez-Rodríguez et al., 2008). Low recovery of swabbed bacteria can remain on the tips of the swabs (Ismaïl et al., 2013; Pérez-Rodríguez et al., 2008). To prevent this from happening, it is essential to mix by vortexing (~30 seconds) to recover all viable microorganisms present on the cotton swabs.

Many studies performed comparison tests between swabbing and contact plate methods (Ismaïl et al., 2013; McEvoy, Nde, Sherwood, & Logue, 2005; Moore & Griffith, 2002; Niskanen & Pohja, 1977; Notermans, Hindle, & Kampelmacher, 1976; Pérez-Rodríguez et al., 2008). By testing both sampling techniques on artificially contaminated surfaces, it was observed that the swabbing method could detect more viable pathogens than contact plating. To date, swabbing methods are the most reliable and effective method used when investigating microbial contamination, particularly in the food industry (Pérez-Rodríguez et al., 2008).

2.10.3 Standard qualitative methods

Standard qualitative methods rely on specific media to isolate viable bacterial cells in food (Mandal, Biswas, Choi, & Pal, 2011). Pathogen detection is based on cultivation (pre-enrichment and enrichment), isolation, screening, and confirmation. Pre-enrichment promotes bacterial growth, and recover injured and stressed cells that were caused by intrinsic (pH, moisture) and extrinsic factors (temperature, oxygen availability) (Baylis, MacPhee, & Betts, 2000; Van der Zee, 1994). While selective enrichment contains antibiotic agents that hinder the growth of non-target organisms and promotes the growth of target organisms (WHO, 2003). After enrichment, colonies were isolated on selective agars by spread plate method. Depending on the agar being used, target colonies were identified by observing colour changes or gas production, which can then be confirmed by biochemical tests (Gracias & McKillip, 2004; WHO, 2003).

2.10.4 Standard quantification methods

Quantitative procedures are used when it is necessary to determine the number of microorganisms present in a sample. This is normally performed by plate count method or the most probable number (MPN) method. Plate count methods are based on culturing dilutions of sample suspensions on agar plates or petrifilm plates, where individual colonies can then be counted visually (Gracias & McKillip, 2004; WHO, 2003). Whilst the MPN method calculates the number of viable organisms in the sample, by preparing decimal dilutions and referencing the result to a standard MPN table. The MPN method is labour intensive and expensive. However, it is observed to be more sensitive than plate count method. MPN is widely used for examining enumeration counts of bacteria below 10 per gram of food (WHO, 2003).

2.10.5 Bacteriological Analytical Manual (BAM) and AOAC Official Methods

The FDA Bacteriological Analytical Manual (BAM) and AOAC Official Methods consists standard methods used for microbial analysis of various foodborne pathogens. Figures 2.2, 2.3, 2.4 and 2.5 summarises the general protocols for the detection/enumeration of *Salmonella, Campylobacter, E. coli*, and *S. aureus.*

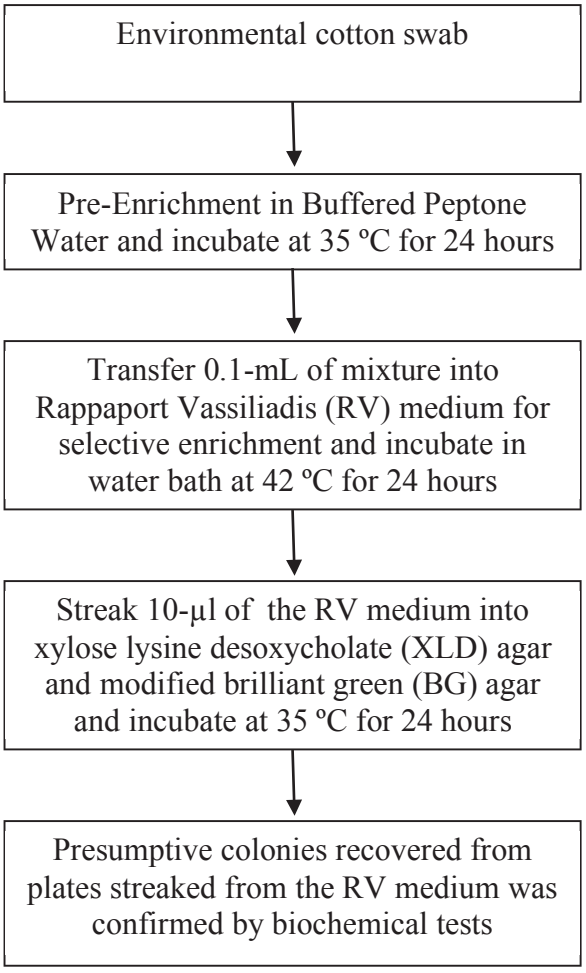

Figure 2.2 Detection procedure of *Salmonella* spp. from environmental swab samples using traditional methods based on BAM
Source: Andrews, Hammack, & Amaguana (2007) and FDA (1998)

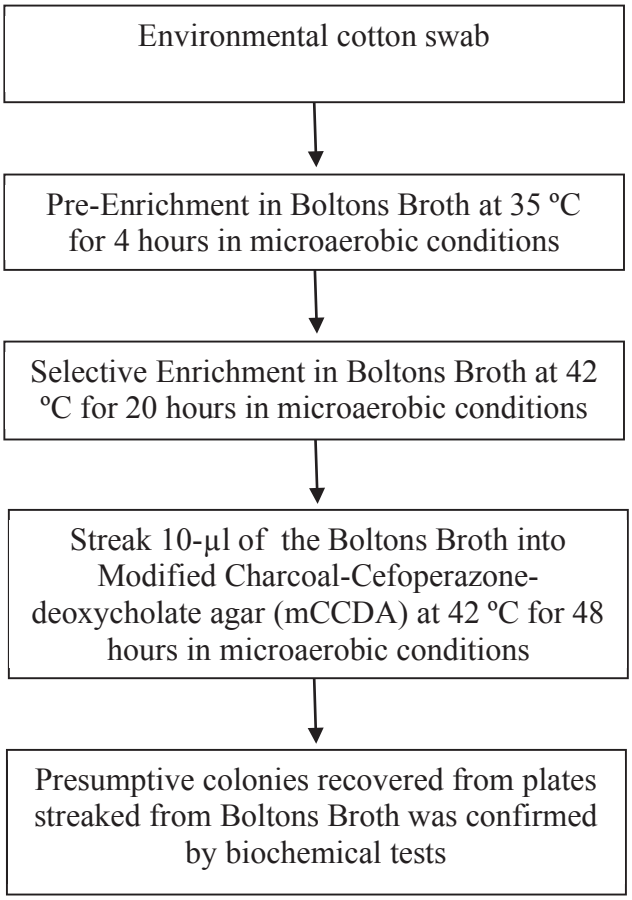

Figure 2.3 Detection procedure of *Campylobacter* spp. from environmental swab samples using traditional methods based on BAM

Source: FDA (1998) and Hunt, Abeyta, & Tran (2001)

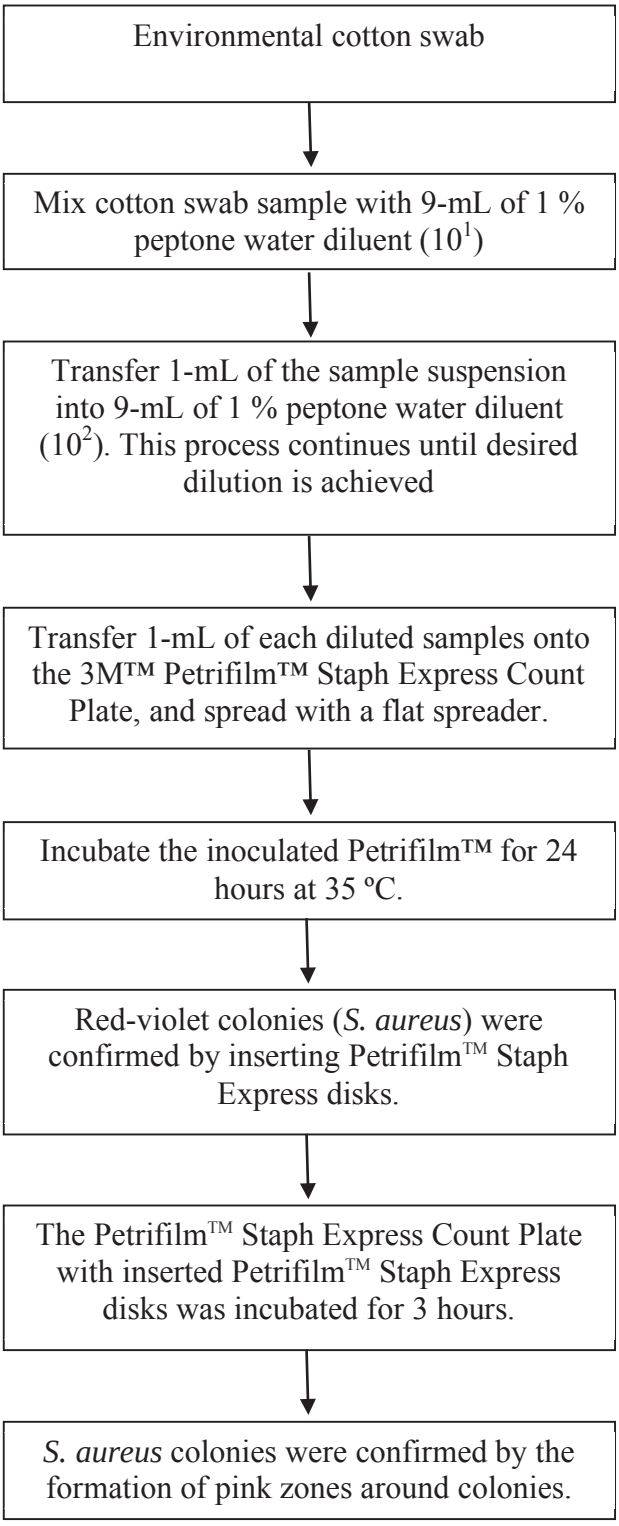

Figure 2.4 Quantification procedure of *S. aureus* from environmental swab samples using traditional methods based on AOAC Official Method 2003.11

Source: Wendy, Victoria, & Ann (2003)

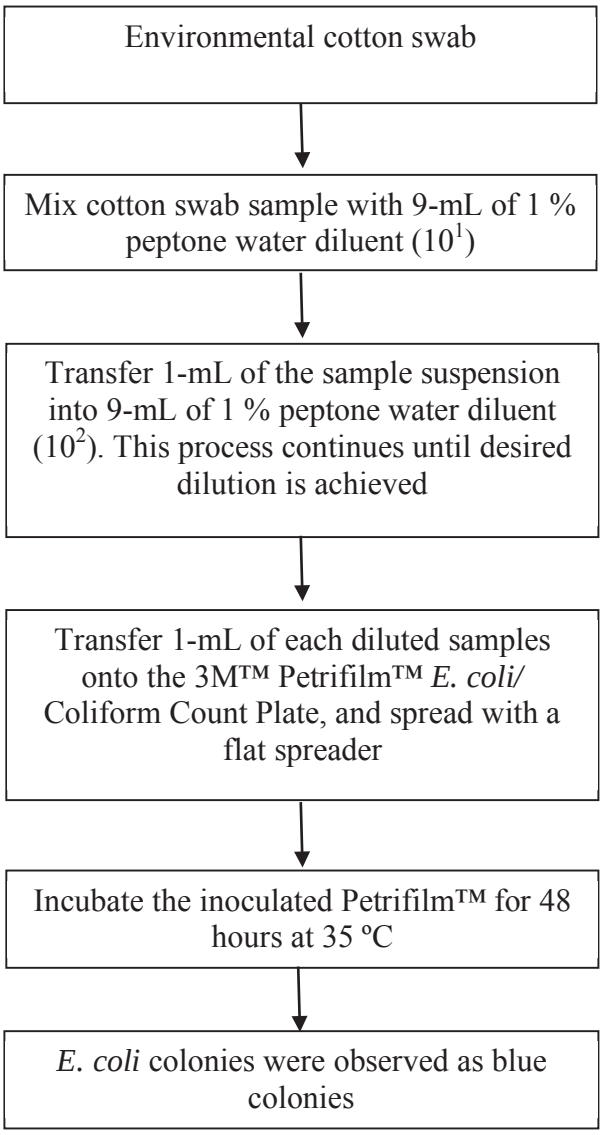

Figure 2.5 Quantification procedure of *E. coli* from environmental swab samples using traditional methods based on AOAC Official Method 991.14

Source: AOAC (1998)

2.10.6 Molecular Detection Methods

Polymerase Chain Reaction (PCR) has become the most commonly used molecular method to determine the presence or absence of foodborne pathogens (Mandal et al., 2011). It specifically detects segments of the DNA that are unique for the identification of species and strain level (WHO, 2003).

PCR is carried out in repetitive cycles of denaturation, annealing, and extension (WHO, 2003). It is performed on DNA obtained by cell lysis and chemical extraction (Rompré, Servais, Baudart, de-Roubin, & Laurent, 2002). During the denaturation step, the double stranded DNA separates into single strands when the temperature is higher than 90 °C. During annealing, the temperature lowers, enabling primers to hybridise to complementary bases of the single strands of target DNA. The DNA polymerase then catalyses the polymerisation of dNTPs to extend two DNA strands from the 3' ends of the primers (Fredricks & Relman, 1999; Rompré et al., 2002). Because the method is performed in repeated cycles, the DNA products amplified in the first cycle, becomes the DNA template for the next cycle, doubling the number of target DNA copies in the reaction (Rompré et al., 2002). The amplification of the PCR cycle can then be assessed by gel electrophoresis (WHO, 2003). In gel electrophoresis, PCR products are loaded into an agarose or acrylamide gel, an electric gradient is applied through a buffer solution, and the PCR products migrates through the gel matrix (Fredricks & Relman, 1999). The gel matrix is stained with nucleic acid stains (EtBr or SYBR green) to visualise the DNA. When a DNA ladder is run on the same gel as the DNA sample, the size of the amplified product can be determined (Van Pelt-Verkuil, Van Belkum, & Hays, 2008). Ideally, a single band of the PCR product is visualised. However, there may be occasions when miss-priming occurs, in which the primers anneal to sites of the genome other than the intended target sequence (Fredricks & Relman, 1999). To determine if the PCR reaction has worked, a positive control is included to ensure there are no non-specific reactions or ambiguities.

PCR assays can undergo multiplexing, where multiple DNA targets are amplified simultaneously in the same reaction tube (Sint, Raso, & Traugott, 2012). In multiplex PCR, several sets of primers are added to the PCR reaction to generate different PCR

products (Fredricks & Relman, 1999; Sint et al., 2012). In this type of assay, the amplicon size of each target must vary, to visualise the specific organism amplified in the reaction through gel electrophoresis (Fredricks & Relman, 1999). For instance, Soumet et al. (1999) developed three sets of primers for the detection of all serotypes of *S. enterica*, and to identify S. Enteritidis and *S.* Typhimurium from environmental swab samples (Soumet et al., 1999).

2.10.7 Molecular Quantification Methods

Real-time polymerase chain reaction (qPCR) is a recent development of PCR. It quantifies specific gene targets in a complex matrix, even at very low concentrations (Arya et al., 2014). The quantification process is achieved by monitoring the fluorescent amplification of a target in real-time (Rompré et al., 2002).

Oligonucleotide probes, designed to hybridise within the target sequence were introduced into the PCR assay for quantification purposes (Arya et al., 2014). The probe was labelled with a reporter fluorescent dye at the 5' end, and a quencher dye attached to the 3' end, to ensure that it could not act as a primer. When the probe is intact, the quencher significantly decreases the fluorescence emitted by the reporter dye (Arya et al., 2014; Juskowiak, 2011; Marras, Tyagi, & Kramer, 2006). But when the target sequence is present, the probe anneals downstream of the primer and is cleaved by the 5'-3' exonuclease activity of Taq (thermostable enzyme) DNA polymerase, allowing the detection of amplified targets due to the fluorescence signal emitted by the probe (Figure 2.6) (Arya et al., 2014; Juskowiak, 2011). This dependence on polymerisation ensures that the cleavage of the probe occurs only if the target sequence was amplified (Van Pelt-Verkuil et al., 2008). After qPCR, the probe fluorescent signal was measured by using chromatography, where the increase in fluorescence intensity is proportional to the amount of PCR products produced during the assay (Arya et al., 2014).

Figure 2.6 Hydrolysis probes during Taq polymerase assay
Source: Arya et al. (2014)

A PCR reaction is broken into three phases: exponential, linear, and plateau (Fraga, Meulia, & Fenster, 2008). The exponential phase begins as soon as the signal of the PCR product becomes greater than the background signal of the assay (Figure 2.7). During this phase, the amount of the DNA target doubles during each cycle, resulting in exponential amplification of the target DNA (Fraga et al., 2008; Van Pelt-Verkuil et al., 2008). As soon as the DNA product accumulates, PCR reaction components become limited, primers begins to compete, and reaction efficiency decreases (Arya et al., 2014; Fraga et al., 2008). The reaction slows down and enters the linear phase, where no doubling of products occurs at the end of each cycle (Figure 2.7). The products formed in this phase varies because of the difference in depletion and accumulation rates (Kubista et al., 2006). The reaction then enters the plateau phase, in which the reaction components becomes depleted and the reaction eventually stops (Figure 2.7). Variation between samples can be observed in this phase due to the differences in the final amount of amplified products (Fraga et al., 2008; Van Pelt-Verkuil et al., 2008).

Figure 2.7 PCR amplification plot at exponential, linear and plateau phase
Note: X axis: cycle number; Y axis: amount of DNA
Source: Fraga et al. (2008)

When measuring amplified products in qPCR, threshold baseline and the threshold cycle (C_t) value is important. Threshold baseline represents a line across a defined range of PCR thermocyclers (3 – 15 cycles), where any amplification signal lies within the "background noise". The threshold baseline affects the calculated C_t value of the assay, because the C_t value is not calculated until the fluorescent signal rises above the background noise of the amplification (Figure 2.8) (Van Pelt-Verkuil et al., 2008). qPCR software automatically calculates the C_t value of the amplification of each sample. The calculated C_t value depends on the amount of amplified target at the exponential phase of the amplification. The lower the concentration, the greater the number of PCR cycles required to increase above the background signal, which in turn, means that the C_t value will be high (Figure 2.8) (Van Pelt-Verkuil et al., 2008).

For absolute quantification, DNA standard curves are used to quantify unknown samples. Absolute standard curves are generated by dilution series of known concentrations of the DNA target (control) (Van Pelt-Verkuil et al., 2008). The C_t values of the diluted standard are then plotted against the DNA concentration (Figure 2.8) (Kubista et al., 2006). Within each PCR run, the C_t value of the unknown sample is calculated against the C_t value of the control (Van Pelt-Verkuil et al., 2008). To manually estimate the concentration of an unknown sample from the qPCR assay, the equation $Y = 10^{((X-c)/m)}$ was established. The equation is based on the linear regression equation generated from the dilution series ($Y= mX + c$), where Y = estimated $\log_{10}$ CFU/mL; c = intercept; X = Ct value of the sample; and m = slope for X (Hu, Hegde, & Lennon, 2012; Kephart & Bushon, 2009).

Figure 2.8 qPCR amplification of DNA serial dilution in a spectrofluorometric thermal cycler
Source: Marras et al. (2006)

The standard curve approach estimates the efficiency of the qPCR assay (Kubista et al., 2006). The amplification efficiency (E) can be calculated by the equation: $E = [10(-1/slope) – 1]$, where 100% efficiency represent the doubling of the product at each cycle. Achieving 100 % efficiency is not always possible because of assay inhibitors (Organic compounds: bile salts, phenol, ethanol, polysaccharides; Proteins: collagen, haemoglobin, immunoglobin G) (Brankatschk, Bodenhausen, Zeyer, & Burgmann, 2012; Fraga et al., 2008; Schrader, Schielke, Ellerbroek, & Johne, 2012). Therefore, optimisation of PCR conditions should be conducted to obtain PCR efficiency between 80 – 115 % (Callbeck et al., 2013; Fraga et al., 2008).

qPCR can also undergo multiplex assays to quantify different target strains (Fraga et al., 2008). The principle behind the multiplex qPCR is the same as multiplex PCR. The target DNA is extracted and mixed in one reaction system in the presence of specific primers, and different labelled probes. The reaction components of the reaction mixture must be adjusted and optimised to ensure equal and stable amplification for all targets (Kubista et al., 2006).

3 Methods and Materials

3.1 Background

Four broiler sheds at a selected poultry farm in Auckland were investigated to determine the efficiency of cleaning and disinfection after the removal of grown birds. The efficiency of cleaning and disinfection protocol between the six-week growth cycle were evaluated three times, from March 2016 to July 2016. Swab samples were collected from the annex floor (service room), fans, vents, metal feed loaders, plastic feeder, plastic drinkers, and wall crevices before cleaning and after disinfection. The cleaning regime used in the poultry sheds comprised of pre-washing, washing, and disinfection.

Pre-washing was done after the removal of used soft wood shavings (litter). This step involved blowing dust and debris out of the sheds from all surfaces at 14.9 m^3/minute using a Back-Pack Blower (BBX7600, Makita, Japan). The washing step of the cleaning regime was achieved by washing the surfaces of the shed with Biostrip 3000™ pre-disinfectant solution (Chemetall, New Zealand Limited). Biostrip 3000™ was applied on the outside (fans, vents) and inside (walls, ceiling, feeders, drinkers, fans, vents) surfaces of the shed using a high-pressure (27.6 k-pascals) water blaster (GX390 High-Pressure Water Blaster; Honda, USA). The pre-washing step was essential to remove all organic soils, oils/fat and biofilm build-up that may affect the performance of the main disinfectant. After 24 hours, the sheds were disinfected (350 mL/m^2) with 3 % Glutasan QCT (Chemetall, New Zealand Limited), where the diluted solution was applied on the inside surfaces of the shed (including fans, vents, feeders, and drinkers) at high pressure (27.6 k-pascals). Disinfected areas were then allowed to air-dry for 24 hours, followed by layering the floor with 50 mm clean soft wood shavings with a tractor. After layering the shavings, the whole shed was then fogged with Glutasen ULV (Chemetall, New Zealand Limited).

The annex (which housed the visitor records, foot bath, temperature and humidity controllers) was cleaned separately from the sheds. The floors, walls, ceiling, and controllers of the annex were hand cleaned (swept using a broom) and disinfected (scrubbed using a broom) with 3% Triton QCT HF (Chemetall, New Zealand Limited).

3.2 Description of the poultry sheds

Each shed (80 by 16.9 m) at the farm had a surface area of 1352 m^2, and was climate-controlled. Each shed was equipped, with an annex (service room), cross ventilation system with 10 fans, 408 feeders, and 1,985 drinkers (Figure 3.1). The average holding capacity of each shed was approximately 20,000 male and female birds.

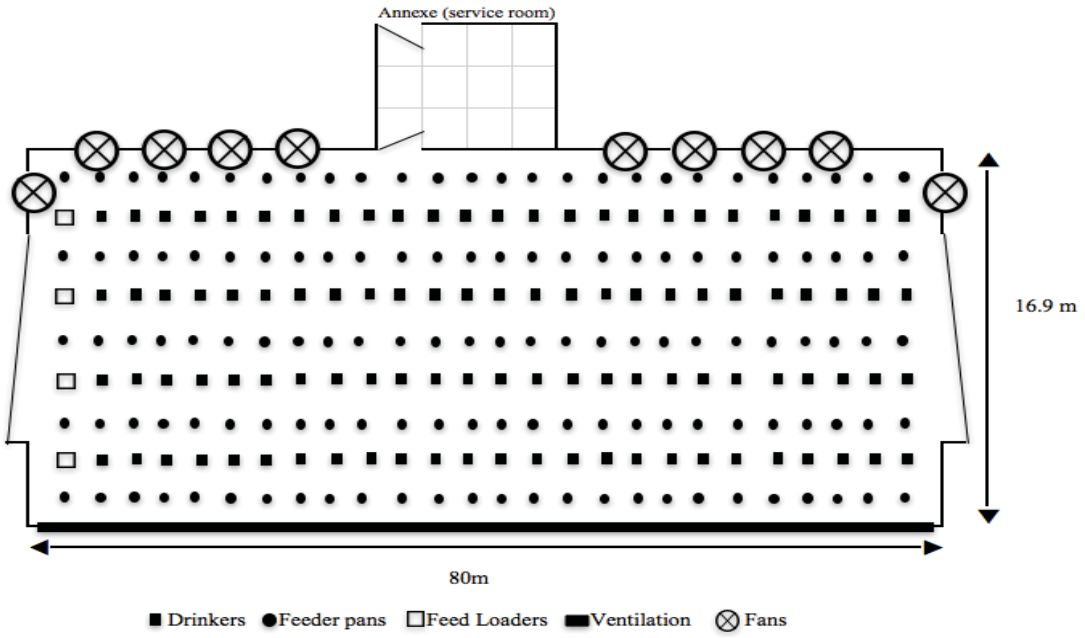

Figure 3.1 Floor plan of broiler shed
Note: Not drawn to scale

3.3 Sampling plan

The sample size used in this study was determined by the epidemiological computer program Win Episcope ® (Thrusfield et al., 2001). Based on previous studies, the minimum estimated prevalence of 50 % was used to generate a sample size with 95 % confidence interval (Castro-Hermida et al., 2015; Kich et al., 2011; Vanantwerpen et al., 2014). In this study, 248 samples were collected in each cycle of the cleaning and disinfection regime. Table 3.1 shows sample numbers generated by Win Episcope ® for each location in the shed.

Win Episcope ® was also used to design the parameters of a simple random sampling plan for each location (Table 3.1). The generated random numbers were used to code feeders, feed loaders, drinkers, fans, vents, and annex floor, which were swabbed. However, as crevices were present on different locations of each shed, it was not possible to generate random numbers for sampling using the software. To keep track on what crevices were sampled, wall crevices located on one area of the shed were swabbed.

Table 3.1 Simple random sampling plan

Location	Samples (n)	Random numbers*
Feeder (blades 1, 3, 7)	5	57, 208, 270, 296, 408
Feed loaders	3	1, 3, 4
Drinkers	5	200, 565, 1060, 1375, 1675
Fans	4	1,4,8,9
Vents	5	21, 27, 37, 67, 74
Annex floor	4	2, 5, 8, 9
Wall crevices	5	NA

Note: * random numbers generated by Win Episcope ®; NA = not applicable

3.4 Collection of swab samples

Swab samples were collected before cleaning (BC) and after disinfection (AD), on the annex floor, fans, vents, feeders, drinkers, and wall crevices. Wet and dry swab sampling protocol of ISO 18593 was used to collect swab samples (ISO, 2004). Firstly, samples were collected by pre-moistening the cotton swab (wet) with buffered peptone water (BPW) (Fort Richard, NZ), and swabbing the surface, vertically and horizontally for 30 seconds. A second cotton swab (dry) was then used to sample over the same surface area, using the same technique. Double swabbing was conducted to optimise collection of bacteria on the surfaces (Beumer, Te Giffel, Spoorenberg, & Rombouts, 1996).

After sampling, each pair of the wet and dry cotton swabs was aseptically broken off into a 15-mL centrifuge tube (Axygen™ sterile; Fisher Scientific Laboratories, USA) that contained one-mL BPW. Swab samples were stored in a cooler box before transporting (~4 ºC) to Massey University at Albany Campus, Auckland for analysis. Samples were processed immediately upon delivery at the Microbiology Laboratory, or stored at 4 ºC

in a refrigerator (Fisher&Pykel, NZ) for no longer than 24 hours (Andrews & Hammack, 2003).

3.5 Sample Preparation

Each pair of wet and dry sample swabs was prepared according to the Bacteriological Analytical Manual (BAM) (Andrews & Hammack, 2003). Nine-mL of 0.1 % peptone water (Fort Richard, NZ) were aseptically added into each swab sample that contained one-mL BPW. The sample suspension ($x10^1$) was mixed thoroughly (30s) with a vortex mixer (VM-96B JEIO TECH, Korea) and then prepared for analysis as shown in Figure 3.2.

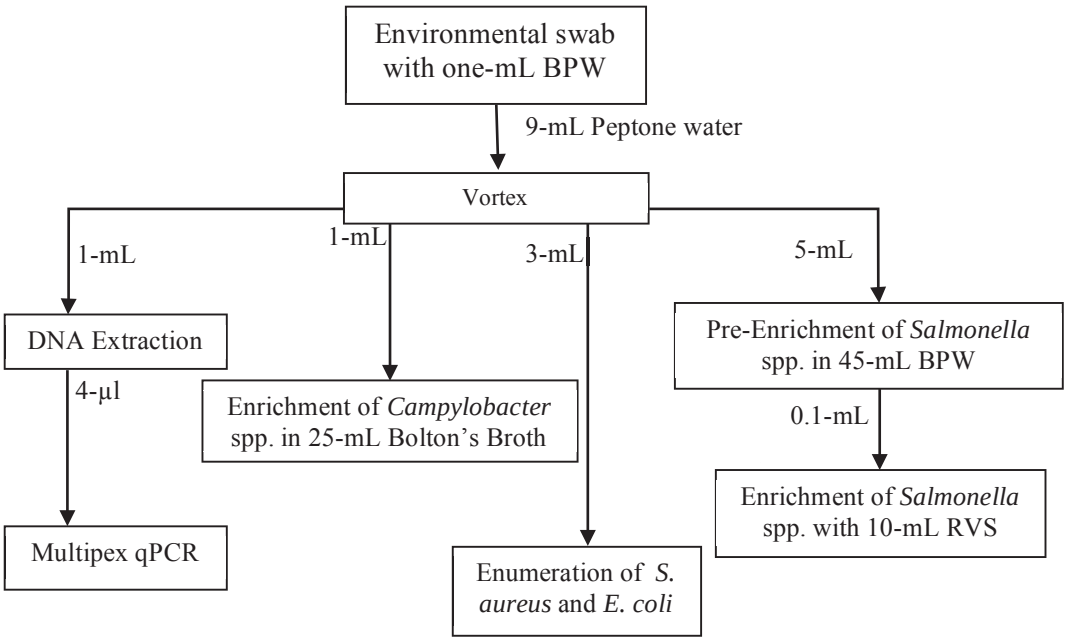

Figure 3.2 Overview of preparation of each swab samples
Note: BPW = Buffered Peptone Water; RVS = Rappaport Vassiliadis Soya; qPCR = Quantitative Polymerase Chain Reaction

One-mL of the cotton swab sample suspension ($x10^1$) was pipetted into a two-mL Eppendorf™ micro-centrifuge tube (Medi'Ray, NZ), and kept at -80 ºC freezer (Heraeus HFU 586 Basic, Germany) until required for real-time PCR analysis (Brankatschk et al., 2012)

For the enrichment of *Campylobacter* spp., one-mL of the cotton swab sample suspension ($x10^1$) was added to a sterile 30-mL screw cap plastic vial (Fort Richard, NZ) containing 25-mL Boltons Broth (Fort Richard, NZ). The prepared vials with samples were loosely capped and carefully placed inside a rectangular plastic container (AnaeroPack™ 7 L; ThermoFisher Scientific, USA). One BBL™ anaerobic indicator strip (Fort Richard, NZ) moistened with potable water, and two CampyGen™ (3.5 L; ThermoFisher Scientific, NZ) sachets was placed inside the container. The container was quickly sealed with an air-tight lid to maintain optimum microaerophilic (84 % N_2, 10 % CO_2, 6 % O_2) conditions (Note: The time taken between opening the sachets, and sealing the container was kept below one minute). The container was incubated for four hours at 35 ˚C, and then transferred to a 42 ˚C incubator for 44 hours. The anaerobic indicator strip was expected to remain blue throughout incubation, to confirm the microaerophilic conditions inside the container.

For the enumeration of *S. aureus* and *E. coli*, one-mL of the cotton swab sample suspension ($x10^1$) was added to nine-mL of 0.1 % peptone water (Fort Richard, NZ) to produce $x10^2$ dilution. The diluted sample ($x10^2$) was mixed thoroughly using a vortex mixer. Serial dilutions of samples were prepared up to $x10^5$ dilution.

For the pre-enrichment of *Salmonella* spp., five-mL of cotton swab sample suspension ($x10^1$) were transferred into a stomacher bag (Fort Richard, NZ) containing 45-mL BPW. The stomacher bag was carefully placed in a plastic tray (making sure the contents did not spill), and incubated at 35 ˚C for 24 hours. The pre-enrichment step was intended to recover injured cells and dilute inhibitory substances present in the sample (Da Silva et al., 2012).

For the selective enrichment of *Salmonella* spp., 0.1-mL of the pre-enriched sample was aseptically transferred into a 10-mL pre-warmed Rappaport Vassiliadis Soya (RVS) broth

tubes (Fort Richard, NZ). The RVS broth tubes were then incubated in a water bath for 24 hours at 42 ˚C. Selective enrichment was conducted to suppress the growth of unwanted bacteria as well as recover injured cells (Da Silva et al., 2012).

3.6 Isolation of *Salmonella* spp. and *Campylobacter* spp. (Standard methods)

Salmonella spp. isolation was based on BAM (Andrews et al., 2007). Ten-µl of enriched sample from the RVS tube were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Fort Richard Laboratories; NZ) and Modified Brilliant Green (MBGA) agar (Fort Richard, NZ) plates. The prepared plates were inverted and stacked into a plastic basket, which was then incubated for 24 hours at 35 ˚C. Developed *Salmonella* colonies on MBG agars were pinkish/red, whereas colonies on XLD agars were observed as pink with black centres (Andrews et al., 2007) Suspected *Salmonella* colonies were confirmed with latex agglutination tests, using *Salmonella* Rapid Latex Test Kit (Microgen®; Ngaio Diagnostics, NZ)

The isolation of *Campylobacter* spp. was based on BAM (Hunt et al., 2001). Ten-µl of the sample, enriched in Boltons Broth (Fort Richard, NZ) were streaked onto Modified Charcoal-Cefoperazone-Deoxycholate (mCCDA) plates (Fort Richard, NZ). The prepared plates were inverted and stacked inside a rectangular plastic container. The container was tightly sealed with an air tight lid, and was incubated under microaerophilic conditions at 42 ˚C for 48 hours. Suspected *Campylobacter* colonies that appeared grey-brown were confirmed with the oxidase (Oxoid™ Oxidase Strips; Fort Richard, NZ) and latex agglutination tests (Microgen® *Campylobacter* Rapid Latex Test Kit; Ngaio Diagnostics, NZ).

3.6.1 Confirmation tests for *Salmonella* and *Campylobacter*

Latex agglutination test was used to confirm the presence of *Salmonella* and *Campylobacter* spp. (Robinson & Batt, 2014). Each oval of the agglutination slide is coated with rabbit immunoglobulin, that was raised against antigen preparations from

selected strains. Once the antibody labelled latex particles were mixed with bacterial suspension, a specific immunochemical reaction occurs, causing the latex particles to agglutinate (Robinson & Batt, 2014).

One drop (~50 µl) of the isotonic saline was dispensed onto the ovals of the agglutination slide following the manufacturer's protocol. A sterile toothpick was used to transfer one to three colonies to two drops of isotonic saline on the slide and was mixed thoroughly to form an even bacterial suspension. One drop of the control latex reagent was then added to one bacterial suspension. Similarly, one drop of test latex was also added to the other bacterial suspension. The bacterial suspensions were mixed thoroughly using a disposable stick supplied with the Rapid Latex Test Kit. The mixtures were then spread to the edges of the oval. After mixing, the slide was rocked gently from side to side for two minutes, keeping the fluid suspensions in constant motion. The agglutination of the test sample indicates the presence of *Salmonella* and *Campylobacter* spp. (Robinson & Batt, 2014).

Oxidase test was also used to confirm the presence of *Campylobacter* spp. The oxidase test works by identifying bacteria that produces the cytochrome oxidase enzyme (FDA, 2001). In the presence of the cytochrome oxidase enzyme, oxidase strips impregnated with N,N-dimethyl-1,4-phenylene diamine and α-naphthol oxidise, forms the coloured compound, indophenol blue (FDA, 2001). The oxidase paper strip was placed in a sterile petri dish (Medi'Ray, NZ). One colony was picked using a sterile toothpick and gently spread onto the test area of the strip. After 10 seconds, a dark blue colour develops, confirming the colony as *Campylobacter* spp. (FDA, 2001).

3.7 Enumeration of *S. aureus* using 3M Petrifilm™ (Standard Methods)

The enumeration of *S. aureus* was based on AOAC Official Method 2003.11 (Wendy et al., 2003), using the Petrifilm™ Staph Express Count Plate (3M™, USA). Petrifilm™ Staph Express Count Plate is a dry rehydratable film that contains chromogenic media (Gracias & McKillip, 2004; Wendy et al., 2003). After preparation of suitable serial dilutions (up to $x10^5$), one-mL of each dilution was pipetted onto the bottom-centre of the Petrifilm™ plate (Figure 3.3). The top film was carefully rolled down to remove air

bubbles, and the solution was evenly distributed with a flat spreader, by gently applying pressure. The prepared Petrifilm™ plates were stacked (maximum 20) and incubated for 24 hours at 35 ˚C. The presence of *S. aureus* colonies was observed as red-violet colonies, due to the presence of a chromogenic ingredient in the medium (Ingham, Becker, & Fanslau, 2003; Wendy et al., 2003).

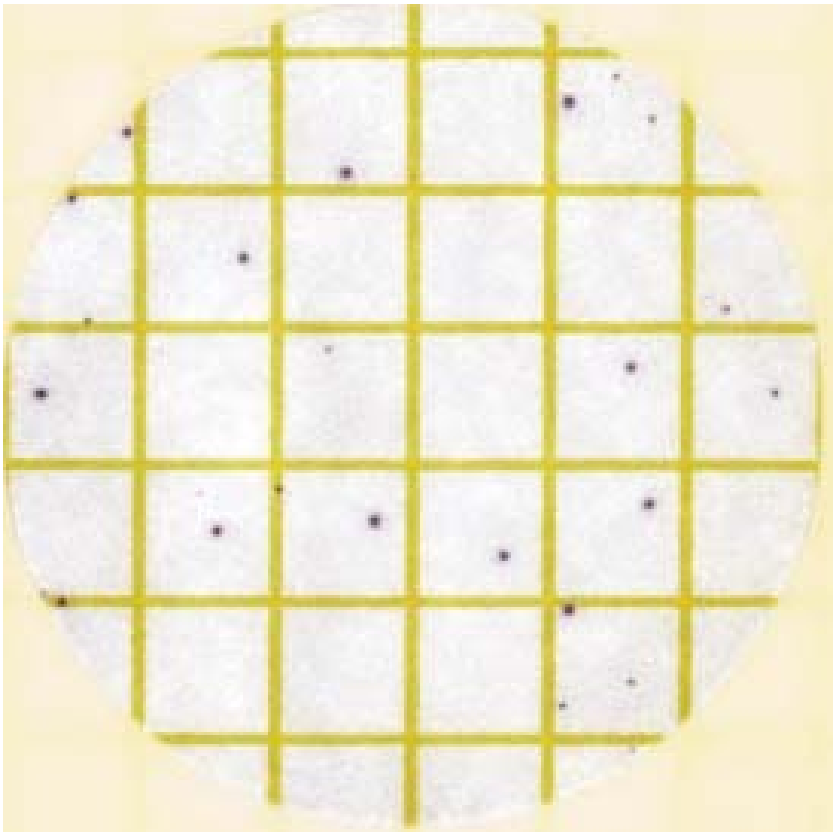

Figure 3.3 Petrifilm™ Staph Express Count Plate with typical red violet *S. aureus* colonies after incubation for 24 hours at 35 ˚C

Source: 3M™ (2014b)

Petrifilm™ Staph Express disks (3M™, USA) were then used to confirm *S. aureus* in other suspected colonies. The disks are made from DNA and toluidine blue-O, to visualise DNase reactions specific to *S. aureus* colonies (Fedio, Wendakoon, Zapata, Carrillo, & Browning, 2008). The top film of the Petrifilm™ was carefully lifted, and the disk was inserted on the well of the count plate. Pressure was then applied on the disk area by

firmly sliding fingers across the top film, ensuring uniform contact of the disk with the gel, as well as removing any air bubbles. The count plates with inserted disks were then stacked (maximum 20) and incubated for three hours at 35 ˚C. The presence of *S. aureus* was identified by the formation of pink zones around the colonies (Figure 3.4).

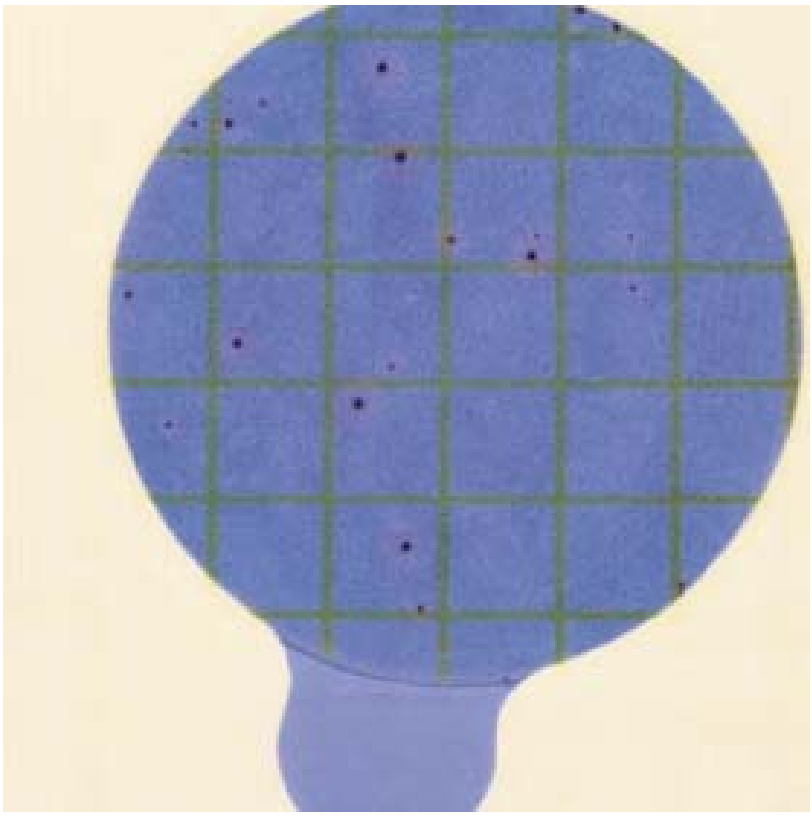

Figure 3.4 Incubated *S. aureus* colonies with typical pink zones on the Petrifilm™, after inserting an express disk

Source: 3M™ (2014b)

3.8 Enumeration of *E. coli* using 3M Petrifilm™ (Standard Methods)

Enumeration of E. coli was based on the AOAC Official Method 991.14 (AOAC, 1998), using Petrifilm™ *E. coli*/ Coliform Count plate (3M™, USA). Petrifilm™ select *E. coli*/ Coliform count plate is a dry rehydratable film coated with nutrients, gelling agents, and a ß-glucuronidase indicator for the identification of *E. coli* (Schraft & Watterworth,

2005). Suitable serial dilutions were prepared (up to x10^5). One-mL of each dilution was plated as previously described for the enumeration of *S. aureus*. The Petrifilm™ plates were incubated for 48 hours at 35 ˚C. The presence of *E. coli* colonies was observed as blue colonies (with or without gas bubbles), due to the ß-glucuronidase activity of *E. coli* that cleaves the chromogenic substrate present in the medium (Figure 3.5) (Schraft & Watterworth, 2005; Warnes & Keevil, 2004).

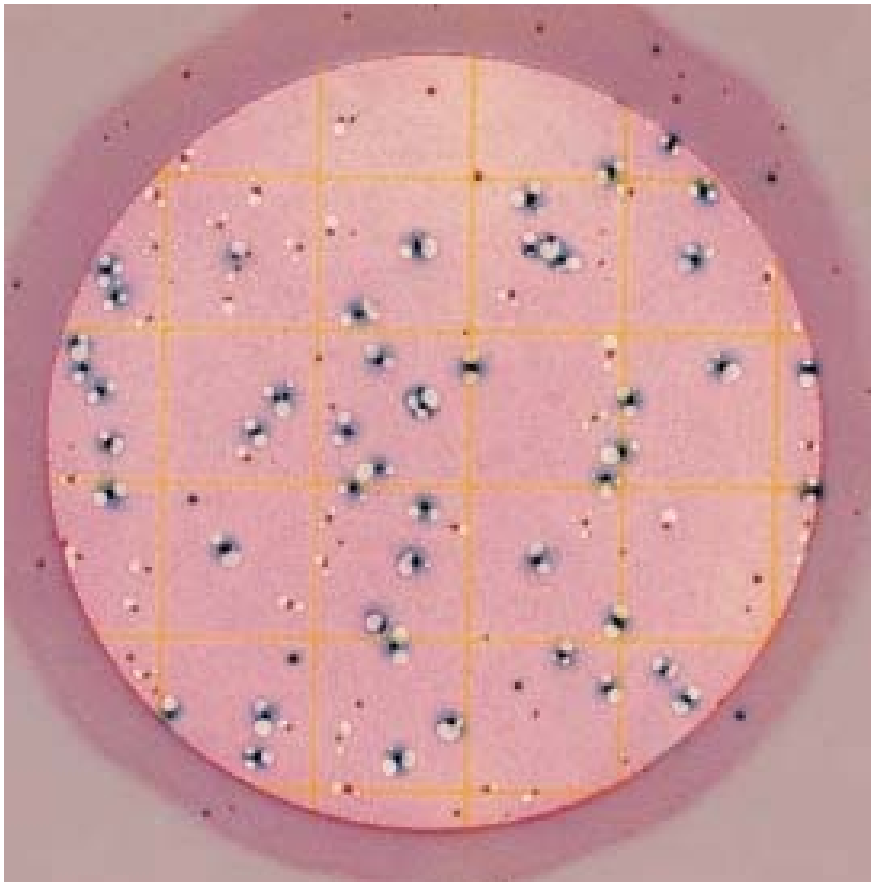

Figure 3.5 Petrifilm™ *E. coli*/ Coliform Count Plate with typical blue colonies (with/ without gas bubbles) after incubation for 48 hours at 35 ˚C

Source: 3M™ (2014a)

3.9 Stock cultures used as positive control for the detection, enumeration and qPCR method

Four bacterial strains from the Institute of Environmental Science and Research Limited (ESR, Porirua, NZ), and the culture collection at the Microbiology Laboratory, Massey University, were used in this study. The specific strains used were *Salmonella* Typhimurium ER94/316, and *Campylobacter jejuni* NCTC 11168, *Staphylococcus aureus* NCTC 4163, and *Escherichia coli* O157 ER93/2637.

3.10 DNA extraction method

Qiagen DNeasy® Blood and Tissue Kit (Bio-Strategy, NZ) were used to extract bacterial genomic DNA from stock culture and test samples. Tests were conducted for *Salmonella* spp. (Gram –ve), *Campylobacter* spp. (Gram –ve), *S. aureus* (Gram +ve), and *E. coli* (Gram –ve). The two types of bacteria (Gram –ve and Gram +ve) contains a peptidoglycan layer (glycan chains crosslinked by peptide bridges) that maintains cell shape, and prevents osmotic lysis. However, Gram +ve cells consists of a thicker peptidoglycan layer, compared to Gram –ve cells (Mahalanabis, Al-Muayad, Kulinski, Altman, & Klapperich, 2009). Therefore, to efficiently extract DNA from both types of bacteria, the DNA extraction protocol for Gram +ve bacteria (based on the manufacturer's instructions) were followed for both culture stocks and samples.

An Eppendorf™ micro centrifuge tube containing one-mL of culture stocks/ cotton swab sample suspension were centrifuged at 5,000 g for 10 minutes (Heraeus™ Pico™ 17 Centrifuge, ThermoFisher Scientific, Germany) to harvest cells for DNA extraction. After centrifugation, the supernatant was carefully discarded, and the bacterial pellet of the culture stocks was re-suspended in 180-µl lysis buffer (20-mg/mL Lysozyme; 20-mM Tris Cl, pH 8.0; 2-mM Sodium EDTA; 1.2 % Triton X-100). The bacterial suspension was incubated at 37 ˚C for one hour on the IsoBlock™ digital dry bath (Benchmark Scientific, USA). Twenty-five-µl Proteinase K and 200-µl Buffer AL (Lysis Buffer) were added to the mixture, mixed by pulse-vortexing (VM-96B JEIO TECH, Korea), and further incubated for one hour at 56 ˚C (with frequent mixing by vortexing). After

incubation, 200-µl of absolute ethanol (Molecular Biology Grade; ThermoFisher Scientific, NZ) was added to the mixture, and mixed by vortexing.

The mixed sample was carefully transferred into the DNeasy® spin column in atwo-mL collection tube (supplied with Qiagen DNeasy® Blood and Tissue Kit), and centrifuged 6,000 g for one minute. The flow through was discarded and the DNeasy® spin column was transferred into a clean two-mL collection tube. Wash Buffer (500-µl, Buffer AW1) was added into the DNeasy® spin column, and centrifuged for one minute at 6,000 g. The flow through was discarded and the DNeasy® spin column was again transferred into a clean two-mL collection tube. Another aliquot of Wash Buffer (500-µl, Buffer AW2) was added into the DNeasy® spin column, and centrifuged for four minutes at 17,000 g. The flow through was discarded, and the DNeasy® spin column was placed into a clean Eppendorf® DNA Lobind micro-centrifuge tube (Medi'Ray, NZ). Fifty (50) µl Buffer AE (Elution Buffer) was added into the DNeasy® spin column, and allowed to incubated for five minutes at room temperature (20 ˚C). After incubation, the DNeasy® spin column was centrifuged for one minute at 6,000 g to elute DNA. The elution step was repeated twice to increase DNA yield of the sample. Once extracted, DNA of culture stocks were stored at 4 ˚C in a refrigerator for short term storage (one week), or -20 ˚C freezer (Fisher&Pykel, NZ) for long term storage (one year).

3.11 Analysis of DNA positive control

The purity and concentration of the DNA extract of the stock cultures were determined by ASP 370 Micro-volume UV/Vis spectrophotometer (ACTGene, USA). The spectrophotometer was calibrated before conducting measurements. Firstly, the upper and lower optical surfaces of the micro-volume spectrophotometer was carefully cleaned with KimWipes (Kimtech Science, USA) moistened with 70 % ethanol (Molecular Biology Grade; ThermoFisher Scientific, USA). Two-µl of Buffer AE (Elusion Buffer from Qiagen DNeasy® Blood and Tissue Kit) were pipetted onto the lower optical surface. The lever arm was lowered, and a blank measurement was performed. After the calibration step, the upper and lower optical surfaces were carefully cleaned with KimWipes moistened with 70 % ethanol. Eppendorf® DNA Lobind micro-centrifuge

tube (Medi'Ray, NZ) containing the DNA extract, was carefully inverted three times to mix the DNA stock. Two-µl of the DNA stock were pipetted onto the lower optical surface. The lever arm was lowered, and the DNA extract was measured to determine DNA purity and concentration of the control stock (Desjardins & Conklin, 2010). The upper and lower optical surfaces were cleaned with KimWipes between measurements. The spectrophotometer was calibrated after every 20 samples to maintain accuracy of the equipment.

DNA concentration of the control stock was determined by the absorbance of the DNA at 260 nm, using a spectrophotometer. In this study, five measurements were conducted to calculate the mean DNA concentration (ng/µl) of each control stock. Once the mean concentration was determined, DNA control stocks were adjusted to 50-ng/µl for plotting the qPCR standard curve and five-ng/µl for PCR, with sterile Milli-Q water (calculations are shown in Tables B2 and B3, Appendix B).

The purity of DNA of the control stocks are shown by the absorbance ratios (260/280 nm) measured by the spectrophotometer. Ratios between 1.8 to 2 indicates pure DNA, whilst absorbance ratios lower than 1.8, may be attributed to protein or phenol contamination (ThermoFisher-Scientific, 2013). The purity of the control stocks used in this study are shown in Tables B1, Appendix B.

3.12 Designing Primers and Probes

Primers and probes are single-stranded DNA sequences (Tang, Morris, Langone, & Bockstahler, 2006). The sequences used in this study were designed by Cremonesi et al. (2014) which specifically targeted: tetrathionate reductase response regulator (ttrR) gene located within *Salmonella* pathogenicity islands; 16S rRNA of *Campylobacter* spp.; heat-shock protein gene (htrA) of *S. aureus*; intimin (eae) virulent genes of *E. coli* O157:H7, respectively (Table 3.2).

Table 3.2 Set of Primers and probes for each microorganism

Microorganism	Target gene	Sequences (5'-3')	Amplicon Size (bp)
Salmonella	ttrR F	CGAAGAGACCCCTGTCGTACT	56
	ttrR R	AAGTGGACGCATCGACCAA	
	ttrR P	**6FAM**-TCGCCGTCGGTATTC-**MGBNFQ**	
Campylobacter	16s rRNA F	CGCCGCGTGGAGGAT	68
	16s rRNA R	GGTACCGTCAGAATTCTTCCCTAAG	
	16s rRNA P	**VIC**-ACGCTCCGAAAAGTGT-**MGBNFQ**	
S. aureus	htrA F	GAAGTAATATCAGACAAATCAAATAC AGTACC	92
	htrA R	TCTTCCGGTAAAGTTAATGGCTTCTG	
	htrA P	**VIC**-CAGATTCCGACAATTTT-**MGBNFQ**	
E. coli O157:H7	Eae F	GTAACAATGTCAGAGGCGAGTTG	73
	Eae R	CCACCGCTTGCTTTCAGTTTAA	
	Eae P	**6FAM**-ATTGCAGCCAAATATT-**MGBNFQ**	

Note: F = Forward Primer; R = Reverse Primer; MGBNFO = Minor Groove Binder Non-Florescent Quencher
Source: Cremonesi et al. (2014)

3.12.1 Preparation of primers and probes

All primers and probes were synthesised by Applied Biosystems® (ThermoFisher Scientific, USA). Primers were supplied in lyophilised form. To prepare a primer stock of 100-µM, the lyophilised primer was pulse-centrifuged (7,000 g) (Heraeus™ Pico™ 17; ThermoFisher Scientific, Germany), and re-suspended in Tris-EDTA (TE) buffer (10-mM Tris, 0.1-mM EDTA, pH 8). The amount of TE buffer added to the lyophilised primers was calculated based on the concentration (nM) provided by Applied Biosystems®. (calculations are shown in Appendix C). The dual labelled TaqMan® minor groove-binder (MGB) probes were supplied in TE buffer at a concentration of 100-µM (stock). The oligonucleotide probes were prepared with reporter dyes on the 5' end, and a non-fluorescent quencher (NFQ) on the 3' end. *Salmonella* spp. and *E. coli* O157:H7 were labelled with reporter dye FAM™ (6-carbocyfluorescein), whist *Campylobacter* spp. and *S. aureus* were labelled with VIC®.

The main primer and probe stock of 100-µM were adjusted to 20-µM with TE buffer. The primer and probe stock were then aliquoted to 50-µl into sterile PCR tubes (Neptune®0.2-mL Flat Cap; Medi'Ray, NZ) and stored at -20 ºC freezer until required (calculations are shown in Appendix D).

When primers and probes were needed for PCR/qPCR analysis, the prepared 20-µM primer and probe stock were thawed on ice in a bucket. Once thawed, primers and probes were adjusted to a working stock of 10-µM (primer) and 5-µM (probe) with TE buffer (calculations shown in Appendix E).

3.13 Optimisation of qPCR assay condition by gradient PCR

According to the report by Cremonesi et al. (2014), the specificity of primers and probes used in this study, only targets *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* 0157:H7. However, gradient PCR was conducted on a T100™ Thermal Cycler (Bio-Rad Laboratories; USA) to optimise assay conditions. Gradient PCR is a function where the instrument can execute different reaction conditions in a single run. In this case, annealing temperatures were adjusted with primer concentrations. A 20-µl reaction was carried out with 10-ng DNA template (2-µl of the 5-ng/µl DNA stock). The reaction mixture of the assay contained 10-µl of Taqman® Gene Expression Master Mix (2x) (ThermoFisher Scientific; USA), 0.9-µM, 0.5-µM or 0.3-µM primer concentrations, and autoclaved milli-Q water (the calculations for making the reaction mixtures can be found in Appendices F and H). Negative control (blank) was included in each experiment, by adding milli-Q water, to monitor the presence of contamination. The amplification was carried out at 50 ˚C for two minutes, 95 ˚C for 10 minutes, followed by 40 cycles at 95 ˚C for 15 seconds, and 52 - 60 ˚C for 60 seconds to anneal/extend DNA.

After gradient PCR, the PCR products were analysed by gel electrophoresis (OWL Scientific, USA). Five (5) percent agarose gel was prepared by dissolving five grams of agarose powder (AppliChem; Germany) in 100-mL 1x Tris-acetate-EDTA (TAE) buffer (40-mM Tris, 20-mM acetate, and 1-mM EDTA). The mixture was heated in the

microwave for two minutes, or until the agarose powder was completely dissolved. Once the gel solution had cooled (~ 40 ˚C), 1x SYBR® safe DNA gel stain (Invitrogen; USA) was added, and mixed thoroughly. The gel mixture was gently poured into the gel-casting tray with a one-mm plastic well-comb, and left to cool at room temperature for 30 minutes or until solidified. Once set, the well-comb was carefully removed and the agarose gel was placed into the gel tank (Note: DNA is negative, it will therefore migrate to the positive end). The gel tank was filled with 1x TAE buffer until the agarose gel was fully covered. Five-µl of each PCR product was mixed with one-µl of 6x Orange DNA Loading dye (ThermoFisher Scientific, USA). Five-µl of the mix was slowly pipetted into each well, and five-µl of the O'RangeRuler 10-bp DNA ladder (ThermoFisher Scientific; USA) was also slowly pipetted into each end of the agarose gel. Electrophoresis was allowed to run using PowerPac™ Basic Power Supply (Bio-Rad; USA) at 95 volts for 1-3 hours. DNA separations (bands) after gel electrophoresis were observed under blue light trans-illuminator (ChemiDoc; Bio-Rad Laboratories), and gel images were captured using the Image Lab ™ software (Bio-Rad Laboratories).

3.14 Sensitivity and specificity of qPCR assays

DNA (controls) standard curves were constructed to confirm the specificity and sensitivity of the qPCR assays. Standard curves based on DNA concentration (pg) were constructed from 10-fold serial dilutions of quantified DNA stocks.

S. aureus, *E. coli*, and *S.* Typhimurium (positive control strains) were streaked onto nutrient agar plates (Fort Richard Laboratories; NZ). The streaked plates were inverted, stacked into a plastic basket, and incubated for 24 hours at 35 ˚C (FDA, 1998). Similarly, C. *jejuni* (positive control strain) was streaked onto mCCDA plates, inverted and stacked inside a rectangular plastic container. The container was tightly sealed, and was incubated microaerophilically at 42 ˚C for 48 hours.

After the bacterial colonies had grown (developed), a loop-full of bacteria was suspended into micro-centrifuge tubes containing one-mL of physiological saline solution (PBS) (0.85 % NaCl; SigmaAldrich, NZ). Bacteria suspensions were mixed thoroughly with a

vortex mixer, and centrifuged (Heraeus™ Pico™ 17; ThermoFisher Scientific, Germany) at 5,000 g for 10 minutes to re-harvest cells (Park et al., 2011). The washing procedure was done three times to remove PCR inhibitors that may be present on agar, before DNA extraction.

Once DNA extraction was performed, DNA purity and concentration were determined. DNA control stocks were adjusted to 50-ng/µl with sterile Milli-Q water. Ten-fold serial dilutions were performed on the 50-ng/µl working stock, until $x10^5$ was obtained. The 10-fold serial dilutions ($x10^5 – x10^1$) corresponded to one to 10,000-pg respectively, when two-µl of each stock were added to a 20-µl qPCR reaction (calculations are shown in Appendix G, Table G1).

3.15 Optimisation of multiplex quantitative PCR

Optimisation of the multiplex qPCR was achieved by adjusting primer concentrations of both targets from 0.8-µM to 0.15-µM, until similar cycle thresholds (C_t) were achieved (< 1 C_t) between qPCR singleplex and multiplex reactions (Al-Tebrineh, Pearson, Yasar, & Neilan, 2012). Calculations can be found in Appendix F, while reaction mixture used for this experiment can be found in Appendices J and K.

3.16 Optimised quantitative singleplex and multiplex PCR amplification conditions

Singleplex qPCR was carried out in a reaction volume of 20-µl. Two-µl of DNA samples/standards was added to a mix containing: 10-µl of Taqman® Gene Expression Master Mix (2x; Applied Biosystems, USA), one-µl of primer (10µM), one-µl of probe (5 µM), and milli-Q water (Appendix I). Negative control was included in each experiment by adding milli-Q water instead of DNA samples/standards. The amplification was carried out at 50 ˚C for two minutes to activate Uracil-DNA Glycosylase (UDG) enzyme, 95 ˚C for 10 minutes to activate AmpliTaq Gold® DNA Polymerase, followed by 40 cycles at 95 ˚C for 15 seconds to denature DNA, and 57 ˚C for 60 seconds to anneal/extend DNA.

For multiplex qPCR reactions, the reaction volume of 20-µl was also used. Four-µl of DNA samples/standards was added to 10-µl of Taqman® Gene Expression Master Mix (2x), one-µl of each primer (10 µM), and one-µl of each probe (5 µM) (Appendix I). Negative control was also included in each experiment by using milli-Q water. The amplification was also carried out at 50 ˚C for two minutes, 95 ˚C for 10 minutes, followed by 40 cycles at 95 ˚C for 15 seconds, and 57 ˚C for 60 seconds to anneal/extend DNA.

All qPCR reactions were performed with StepOne™ Real-Time PCR System (Applied Biosystems, USA). The Real-Time PCR System included a StepOne™ software that measured fluorescence signals during amplification. During amplification, a fluorescence signal amplified by the reporter dye (FAM™ or VIC®) was measured against a passive reference dye (ROX™) signal to normalise fluorescence fluctuations occurring during each amplification cycle (Welti et al., 2003). The C_t value of each sample could then be calculated when the fluorescence of the reporter dye had exceeded the threshold limit (background fluorescence) (Welti et al., 2003). When the fluorescence signal increased over 40 cycles (C_t 40), the sample was considered as negative (Gordillo, Rodriguez, Werning, Bermudez, & Rodriguez, 2014).

3.16.1 Validation of multiplex qPCR using unequal DNA concentrations

Ten-fold serial dilution experiments were conducted to determine the accuracy of the multiplex qPCR assay, when one target (*Salmonella* spp./ *Campylobacter* spp.) was in excess. DNA concentration of one target was kept consistent at 10,000-pg, whilst the other target (*Salmonella* spp./ *Campylobacter* spp.) had varying DNA concentrations, ranging from one to 10,000-pg (Eckford-Soper & Daugbjerg, 2015). These were then analysed by using optimised multiplex qPCR reaction conditions.

3.16.2 Validation of multiplex qPCR in environmental samples

To determine the presence of PCR inhibitory substances in the extracted DNA samples, standard curves were constructed using inoculated environmental samples. Bacterial dilutions prepared from pure cultures were inoculated in environmental samples that are *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* 0157:H7, negative (Eckford-Soper & Daugbjerg, 2015).

A loop-full of *S.* Typhimurium, *S. aureus*, and *E. coli* colonies (from prepared positive control plates), was each transferred into separate 15-mL centrifuge tubes (Axygen™ sterile; Fisher Scientific, USA) containing 15-mL of Brain Heart Infusion (BHI; Fort Richard Laboratories NZ) broth. The tubes were loosely capped, placed on a plastic stand, and then incubated for 20 hours at 35 ˚C. Similarly, a loop-full of *C. jenuni* colonies, was transferred into plastic vials (Fort Richard, NZ) containing 25-mL of Boltons Broth (Fort Richard, NZ). The vials were loosely capped, and placed inside a rectangular plastic container with an anaerobic indicator strip. The container was tightly sealed and incubated microaerophilically for four hours at 35 ˚C, and then 16 hours at 42 ˚C.

After incubation, 10-fold serial dilutions were prepared to determine the concentration of each bacterial stock. One-mL of bacterial suspension ($x10^0$) was added to nine-mL of 0.1 % peptone water, to produce $x10^1$ dilution. The diluted sample ($x10^1$) was mixed thoroughly using a vortex mixer. Several serial dilutions were prepared (up to $x10^7$). Diluted samples (0.1-mL of $x10^5$ - $x10^7$ dilutions) from the stock cultures of *S.* Typhimurium, *S. aureus,* and *E. coli* were spread-plated on solidified nutrient agar (Fort Richard, NZ) plates, whilst *C. jejuni* was spread-plated on Columbia Sheep Blood Agar (Fort Richard, NZ) plates.

One-mL of each dilution ($x10^1$ - $x10^7$) of the stock cultures was inoculated in environmental samples that were confirmed to be free of *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* 0157:H7 by using standard methods. The sample suspension was then set aside for DNA extraction, which was conducted in triplicate to account for DNA loss. These were then analysed by using the optimised multiplex qPCR reaction conditions.

3.17 Method comparison between plate count and qPCR method

One hundred environmental samples analysed by standard methods were also analysed by using the qPCR method. The standard curve generated from inoculated samples (section 3.16.2) was used to calculate the concentration (Log_{10} CFU/mL) of *S aureus* from the C_t values obtained by qPCR (Macé et al., 2013). All qPCR samples were carried out in triplicate, and the mean values were calculated. The comparison between the concentration of *S. aureus* acquired by the plate count and qPCR method was determined by using Bland-Altman non-parametric test of difference, and a scatter-plot with an identity line (Botaro et al., 2013).

4 Statistical analysis

The prevalence and microbial load of sampling sites were analysed using the Statistical Package for Social Sciences (SPSS; Version 19.0, IBM corporation, New York, USA). The prevalence of each bacterium on vents, fans, drinker, feeder, feed loader, annex and crevices for each sampling time was determined by cross-tabulation. All bacterial colony counts were transformed to Log_{10} CFU per mL, and reported as means. The detection limit of the enumeration data was 1 Log_{10} CFU/mL. To visualise the data, stacked bar graphs were generated using the SPSS software.

Standard curves were constructed by performing linear regression analysis (Microsoft Excel 2011; Microsoft Corporation, Redmond, WA) on threshold cycle (C_t) values against pg or Log_{10} CFU/mL of each dilution series (Malorny, Lofstrom, Wagner, Kramer, & Hoorfar, 2008). The best fit of the standard curve was determined by the correlation coefficient (R^2), where $R^2 > 0.98$ indicated the best fit of the standard curve. Amplification efficiency (E) was also calculated, where E = [10 (-1/slope) – 1]. In this study, efficiency between 80 % - 115 % was considered acceptable (Callbeck et al., 2013). However, assay efficiency may only vary by < 5 % between singleplex and multiplex assays (Eckford-Soper & Daugbjerg, 2015).

To estimate the concentration (Log_{10} CFU/mL) of an unknown sample from the qPCR assay, equation x was used based on the linear regression equation (Y = mX + c), where Y = estimated Log_{10} CFU/mL; c = intercept; X = C_t value of the sample; and m = slope for X (Hu et al., 2012; Kephart & Bushon, 2009; Yang, Jiang, Huang, Zhu, & Yin, 2003).

$$Y = (10^{((X - c)/m)}) \times 10 \ldots\ldots\ldots\ldots\ldots\ldots [x]$$

To determine the agreement between the quantitative results for the plate count method and the proposed qPCR method, the Bland-Altman non-parametric test of difference of SPSS was used. A scatter-plot with an identity line (y=x) was also used to determine the agreement between the methods.

5 Results and Discussion

5.1 Introduction

Poultry has been reported to be the most important reservoir for causing most food poisoning incidents worldwide (Sanders, 1999). Previous studies observed that the prevalence of pathogens in bird and environmental samples on the farm, was significantly associated with the prevalence observed in carcass rinse from the same flocks during processing (Berghaus et al., 2013; Schroeder, Eifert, Ponder, & Schmale, 2014; Volkova et al., 2010). Consequently, effective management practices on the farm would reduce contamination at processing, and of the final products.

In this study, three cleaning and disinfection regimes were evaluated between a six-week growth cycle, from March 2016 to July 2016. A total of 248 environmental swab samples located on different areas of the shed (annex floor, wall crevices, plastic drinkers, metal feed loaders, plastic feeder, fans, and vents), were collected from four sheds, during each cleaning and disinfection schedule. The samples were prepared for the detection of *Salmonella* spp. and *Campylobacter* spp. by sample enrichment, isolation, and identification. Bacterial prevalence before cleaning and after disinfection over the three consecutive cycles were analysed through cross-tabulation. The samples were also prepared for the enumeration of *S. aureus* and *E. coli*, by serial dilution and plating. Samples enumerated for each location before cleaning and after disinfection, over three consecutive cycles were reported as means. In addition, multiplex qPCR was developed and validated to enumerate *Salmonella* spp. and *Campylobacter* spp. positive samples.

Due to the nature of field trials, uncontrolled variables may be not unexpected. The uncontrolled variables may include heterogeneity of contamination levels (Luyckx et al., 2015; Newell & Fearnley, 2003), seasonal variability of pathogen prevalence, which may be higher during warmer climates (Allen & Newell, 2005; Evans & Sayers, 2000; Refregier-Petton, Rose, Denis, & Salvat, 2001), and different cleaning teams operate with different standards (Refregier-Petton et al., 2001).

5.2 Detection of pathogens using standard methods

5.2.1 Annex

Pathogen control measures are an integral part of farming operations aimed at preventing the infection of livestock by pathogens (Allen & Newell, 2005). As reported in previous studies, human activity can easily introduce pathogens into the shed, which may infect flocks (Allen & Newell, 2005; Guerin et al., 2007; Marin, Balasch, Vega, & Lainez, 2011). Therefore, hygiene barriers are important, not only for farmers, but also for other personnel (Robyn et al., 2015).

An annex shed is a facility that allows farm workers to sanitise their hands and use foot baths before tending to the birds (Allen & Newell, 2005). Although several hygiene parameters have been reported (Allen & Newell, 2005; Evans & Sayers, 2000; McDowell et al., 2008), the sanitation of the annex shed has not been previously investigated.

Ninety-six swab samples were collected on the annex floor before cleaning and after disinfection, over three consecutive cycles of the cleaning regime (Figure 5.1). Cross-tabulation were used to analyse samples for the prevalence of *Salmonella* spp. and *Campylobacter* spp. (McDowell et al., 2008). Prior to cleaning, the prevalence of *Salmonella* spp. in cycles 1, 2, and 3 were 31.3 % (5/16), 18.8 % (3/16), and 6.3 % (1/16). Similar decreases were also observed between the respective cycles during the analysis of *Campylobacter* spp. with 31.3 % (5/16), 25 % (4/16), and 6.3 % (1/16) prevalence (Figure 5.1). The steady decrease of the prevalence of bacteria between each cycle before the cleaning regime may be due to seasonal variability. Previous studies reported that flock infection peaked in the summer and autumn months, compared to winter and spring (Lara & Rostagno, 2013; McDowell et al., 2008; Refregier-Petton et al., 2001). For instance, Vandeplas et al. (2010) reported a seasonal effect of *Campylobacter* spp. prevalence, where 33.3 % and 100 % were observed during the winter and summer months, respectively. Meanwhile, Schulz et al. (2011) reported no significant seasonal effect on the prevalence of *Salmonella* spp. However, Schulz et al. (2011) concluded that seasonal variations may be just a trend, where a possible relationship between

temperature and pathogen transmission exists. In this study, *Salmonella* spp. was not detected in cycle 1 and 2, but were detected in cycle 3 (6.3 % (1/16) prevalence) after disinfection. Whereas *Campylobacter* spp. was detected in cycles 2 and 3 after disinfection with 6.3 % (1/16) prevalence on both cycles. The results suggest that the cleaning regime of the annex decreased the prevalence of *Salmonella* spp. and *Campylobacter* spp. (Figure 5.1).

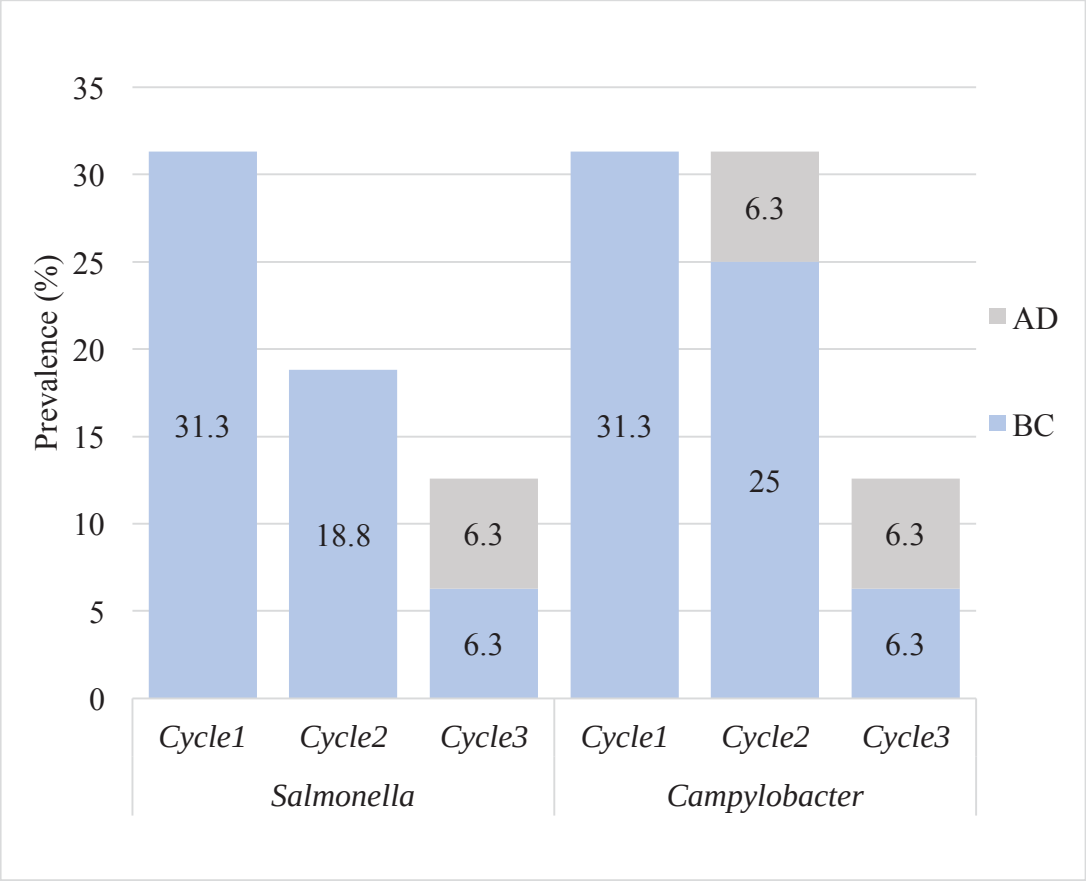

Figure 5.1 Prevalence of *Salmonella* and *Campylobacter* spp. in the annex during three consecutive cycles of cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Swab samples (n = 96) collected on the annex floor were also analysed for *S. aureus* and *E. coli* viable cell counts (Table 5.1). Mean viable cell counts of *S. aureus* obtained before cleaning during the three cycles, varied from 4.36 to 5.68 Log_{10} CFU/mL. After disinfection, a decrease in mean counts was observed in cycles 1, 2, and 3 at 1.27, 2.47,

and 1.49 Log_{10} CFU/mL respectively. Meanwhile, mean cell counts of *E. coli* (3.73 to 1.89 Log_{10} CFU/mL) were lower before cleaning. And it seems as though the cleaning regime could reduce *E. coli* cell counts in cycles 1 and 2 by 1.17 and 0.53 Log_{10} CFU/mL. The decrease of mean cell counts for *S. aureus* and *E. coli* observed after disinfection, was not significant to conclude efficient cleaning. The presence of high microbial counts before cleaning, could have affected the efficacy of the disinfectant used (Ray & Bhunia, 2007). However, the increase of *E. coli* cell count (1.63 Log_{10} CFU/mL) in cycle 3 after disinfection may be due to cross contamination by farm workers, indicating the importance of having hygiene barriers before entering the main shed (Table 5.1). Previous studies have indicated that boots worn by personnel at the farm are possible sources of contamination, especially when the boots had contact with soil and animal excreta. This may be applicable to most pathogens, and not only for *E. coli* (Friese et al., 2013; Herman et al., 2003; Locking et al., 2001; Rose et al., 2000).

Table 5.1 Mean prevalence and counts of *S. aureus* and *E. coli* of samples collected at the annex

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	15 (93.8)	14 (87.6)	4.92 (0.48)	3.73 (1.08)
	AD	14 (87.5)	3 (18.8)	3.65 (0.91)	2.56 (0.85)
2	BC	16 (100)	9 (56.3)	5.68 (0.66)	2.24 (0.22)
	AD	3 (18.8)	2 (12.5)	3.21 (1.45)	1.71 (1.00)
3	BC	14 (87.5)	5 (31.3)	4.36 (0.55)	1.89 (0.71)
	AD	14 (87.5)	1 (6.3)	2.87 (1.43)	3.52 (NA)

Note: NA = not applicable; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

5.2.2 Crevices

Organic matter can accumulate in crevices, which can protect pathogens from chemical agents and then contribute to residual contamination or biofilm formation (Allen & Newell, 2005). Most broiler sheds are made of concrete, and when not properly maintained, crevices become apparent (Allen & Newell, 2005). It is therefore important that the shed is properly cleaned so that organic materials are washed away before disinfection (Mueller-Doblies, Carrique-Mas, Sayers, & Davies, 2010). Inefficient disinfection of crevices can be improved by spot treatment with double strength disinfection (Davies, Breslin, Corry, Hudson, & Allen, 2001). Additionally, the risk of flock infection can be reduced by patching up crevices (Luyckx et al., 2015).

Swab samples (n = 120) were collected from wall crevices before cleaning and after disinfection, over three consecutive cycles of the cleaning regime. The samples were analysed for the prevalence of *Salmonella* spp. and *Campylobacter* spp. through cross-tabulation (Figure 5.2). Before cleaning, the prevalence of *Salmonella* spp. was 60 % (12/20), 5 % (1/20), and 15 % (3/20) in cycle 1, cycle 2, and cycle 3. Compared to the prevalence of *Salmonella* spp., *Campylobacter* spp. were lower (35 %, 7/20) in cycle 1; whilst cycle 2 (10 %, 2/20) and 3 (30 %, 6/20) were observed to have higher prevalence. After disinfection, prevalence of *Salmonella* spp. and *Campylobacter* spp. were similar in cycles 1 and 3 at 30 % (6/20) and 10 % (2/20), respectively. However, in cycle 2, the prevalence of *Campylobacter* spp. was 5 % (1/20), whilst *Salmonella* spp. was not detected. The high prevalence of *Salmonella* spp. and *Campylobacter* spp., observed after disinfection in cycle 1, compared to cycles 2 and 3, may be due to organic residues not efficiently removed during cleaning. Mueller-Doblies et al. (2010) reported similar results during their investigation involving floor cracks. As discussed earlier in this section, when organic matter or biofilms remain after power-washing, the disinfectant used can be deactivated, resulting in the retention of higher bacterial counts. High bacterial concentration observed before cleaning in cycle 1 could have been affected by the application of the disinfectant used in the previous cycle.

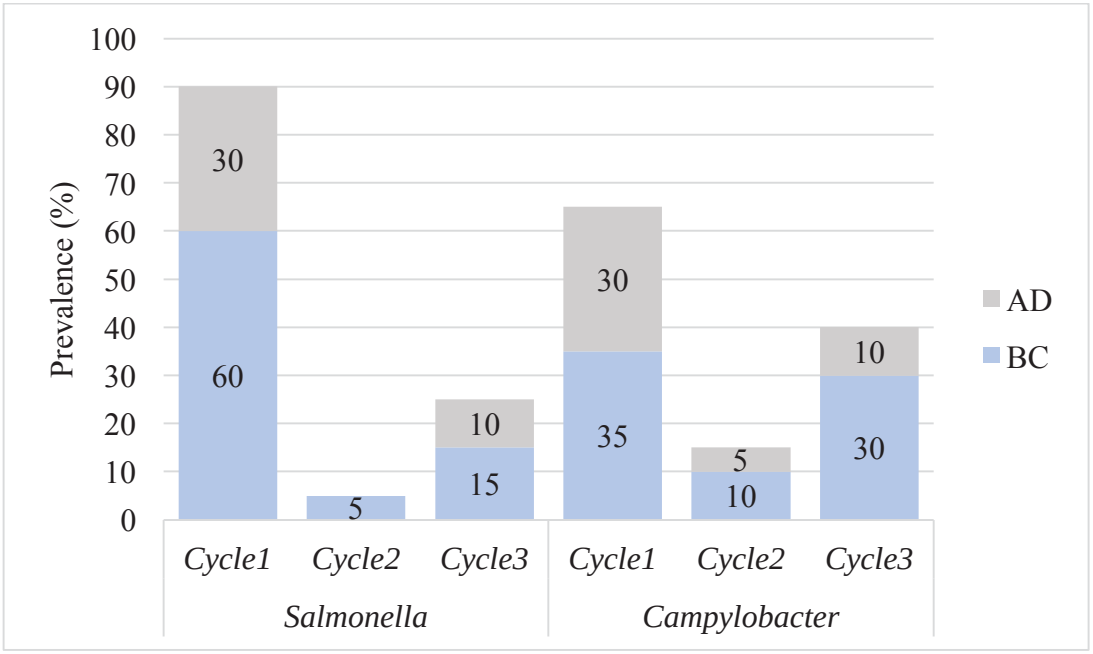

Figure 5.2 Prevalence of *Salmonella* and *Campylobacter* spp. in crevices during three consecutive cycles of the cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Swab samples (n = 120) from wall crevices were also analysed for *S. aureus* and *E. coli* counts (Table 5.2). The mean counts of *S. aureus* before cleaning ranged from 6.07 to 5.15 Log_{10} CFU/mL. Whilst after disinfection, a decrease in cell counts were observed in cycles 1, 2 and 3 at 1.5, 0.65 and 2 Log_{10} CFU/mL respectively. Meanwhile, the mean counts of *E. coli* before cleaning, were 3 Logs lower than the mean counts of *S. aureus*, which ranged from 3.71 to 2.73 Log_{10} CFU/mL. A slight decrease in *S. aureus* mean counts were then observed after disinfection; where the counts decreased by 0.94 Log_{10} CFU/mL in cycle 1, 0.35 Log_{10} CFU/mL in cycle 2, and 1.24 Log_{10} CFU/mL in cycle 3. The decrease of mean cell counts for *S. aureus* and *E. coli* obtained after disinfection, were not significant, probably due to the presence of organic residue in crevices. As reported by Luyckx et al. (2015), cracks are one of the areas that may be difficult to clean due to poor access. Additionally, excess water present in crevices after washing may dilute the disinfectant, which can reduce the efficacy of the disinfectant used.

Table 5.2 Mean prevalence and counts of *S. aureus* and *E. coli* of samples collected in crevices

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	17 (85)	14 (70)	5.27 (1.05)	3.71 (0.68)
	AD	16 (80)	6 (30)	3.77 (1.34)	2.77 (0.90)
2	BC	17 (85)	19 (95)	6.02 (1.81)	2.81 (0.65)
	AD	7 (35)	10 (50)	5.37 (1.18)	2.46 (0.86)
3	BC	18 (90)	13 (65)	5.15 (0.92)	2.73 (0.77)
	AD	17 (85)	3 (15)	3.15 (0.83)	1.49 (0.85)

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

5.2.3 Drinkers

Poultry drinking water has been implicated as a risk factor for the infection of birds by pathogens (Pearson et al., 1993). Thick biofilms can form on water systems, thus affecting water quality (Pearson et al., 1993). It is therefore important to regularly clean and disinfect the water tank and the whole water system to eliminate biofilm formation, to reduce the risk of pathogen colonisation (Allen & Newell, 2005; J. Cox & Pavic, 2010; Evans & Sayers, 2000; Luyckx et al., 2015).

Therefore, to assess contamination on drinkers, 120 swab samples were collected before cleaning and after disinfection over three consecutive cycles of the cleaning regime (Figure 5.3). The samples were analysed to determine the prevalence of *Salmonella* spp. and *Campylobacter* spp. in drinkers through cross-tabulation.

In cycle 3, the prevalence of *Salmonella* spp. and *Campylobacter* spp. were 5 % (1/20) and 15 % (3/20) before cleaning, respectively. After disinfection, *Salmonella* spp. and *Campylobacter* spp. was not detected, indicating that the cleaning regime was efficient, especially when low bacterial prevalence was observed before cleaning. Since drinkers are frequently contaminated by organic matter during rearing, low prevalence of bacteria on drinkers before cleaning are not frequently observed (Poppe, Irwin, Messier, Finley, & Oggel, 1991).

In cycles 1 and 2, prevalence of *Campylobacter* spp. was 50 % (10/20) before cleaning. Whereas, after disinfection, prevalence of *Campylobacter* spp. was 30 % (6/20) and 10 % (2/20), respectively (Figure 5.3). The difference in the prevalence of *Campylobacter* spp. after disinfection in cycles 1 and 2, despite having similar prevalence before cleaning, may be attributed to cleaning standards by different cleaning teams**.** Meanwhile, the prevalence of *Salmonella* spp. in cycle 1 and 2 before cleaning were 45 % (9/20) and 35 % (7/20). Whilst **t**he prevalence of *Salmonella* spp. decreased after disinfection to 20 % (4/20) and 15 % (3/20), respectively (Figure 5.3). The prevalence of both *Campylobacter* spp. and *Salmonella* spp. after disinfection, obtained in cycles 1 and 2, may be explained by the presence of residual organic matter containing pathogens after disinfection. As reported by Battersby, Walsh, Whyte, and Bolton (2017), ineffective cleaning and disinfection may be due to a range of factors including ineffective concentration/ dilution of disinfectants, unhygienic design of equipment, failure to remove organic matter prior to disinfection, following inadequate procedures, short contact time, and immediate recontamination.

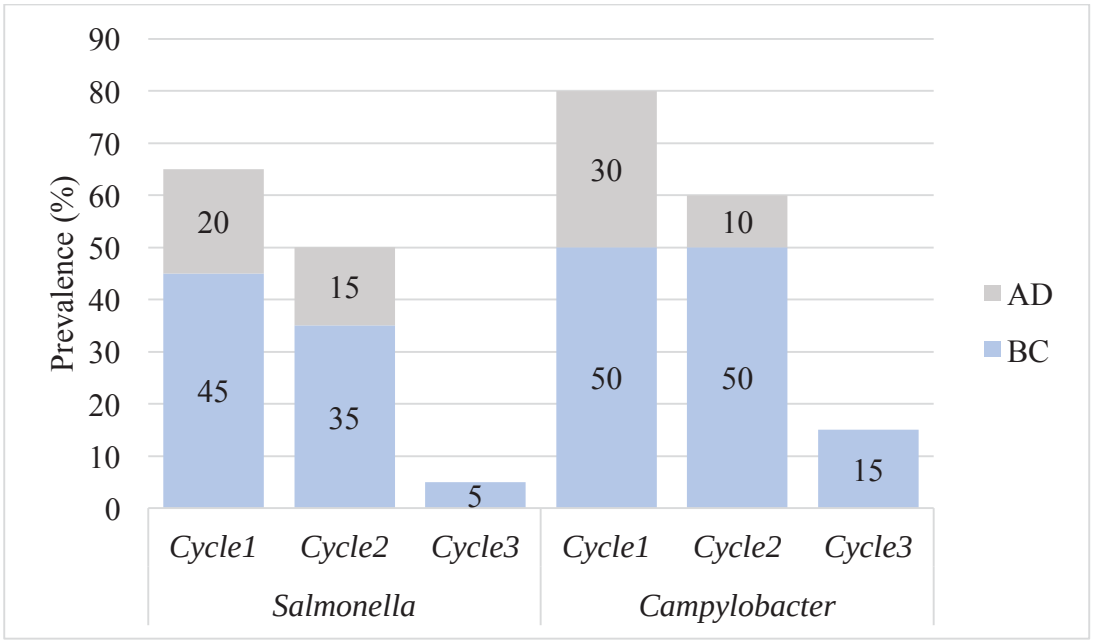

Figure 5.3 Prevalence of *Salmonella* and *Campylobacter* spp. in drinkers during three consecutive cycles of the cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

The collected swab samples (n = 120) were also analysed for viable cell counts of *S. aureus* and *E. coli* on drinkers (Table 5.3). Mean counts of *S. aureus* before cleaning ranged from 6.2 to 3.93 Log_{10} CFU/mL. Mean counts of *E. coli* (4.6 to 2.17 Log_{10} CFU/mL) were 1 to 2 Logs lower than mean counts of *S. aureus*. Results suggest that the cleaning regime decreased cell counts of *S. aureus* by 1.46 (cycle 1), 0.7 (cycle 2), and 1.59 (cycle 3) Log_{10} CFU/mL. Meanwhile, the results indicate that *E. coli* were successfully removed on drinkers after disinfection in cycle 3. However, in cycles 1 and 2, mean counts of *E. coli* were observed to decrease by only 2.05 and 0.81 Log_{10} CFU/mL, respectively. Despite having standard sanitation protocols, the Log_{10} decrease after cleaning was not consistent, which may be explained by discrepancies in cleaning standards of by cleaning teams. Additionally, the sensitivity and efficacy of the disinfectant used were observed to be dependent on the bacterial concentration observed before cleaning. In this regard, since cell counts of *E. coli* were lower before cleaning, the mean counts of *E. coli* had a higher Log_{10} decrease after performing the cleaning regime, compared to the mean counts of *S. aureus* after disinfection. Bower and Daeschel (1999) reported similar results and concluded that the penetration of disinfectants is less effective when aggregation of bacterial cells is present.

Table 5.3 Mean prevalence and counts of *S. aureus* and *E. coli* of samples collected in drinkers

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	19 (95)	18 (90)	6.20 (0.60)	4.60 (1.54)
	AD	19 (95)	8 (40)	4.74 (0.80)	2.55 (1.24)
2	BC	19 (95)	19 (95)	5.22 (1.29)	3.35 (1.19)
	AD	10 (50)	8 (40)	4.52 (0.80)	2.54 (0.74)
3	BC	20 (100)	8 (40)	3.93 (0.92)	2.17 (0.71)
	AD	9 (45)	ND	2.34 (1.24)	ND

Note: ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

5.2.4 Feed loaders and feeders

Feed serves as an indirect route of bacterial transmission to poultry flocks (Maciorowski, Jones, Pillai, & Ricke, 2007). Therefore, several studies have investigated pathogen contamination routes of feed (Friese et al., 2013; Maciorowski et al., 2007; Marin et al., 2011). Marin et al. (2011) reported the prevalence of bacteria in feed collected directly from trucks (2 %) and feeders (17 %). Feeders are therefore important sources of feed contamination, especially when contaminated dust are present after disinfection. Friese et al. (2013) reported high prevalence of *S. aureus* (50 %) on feed samples collected from feeders. In their study, Friese et al. (2013) observed that feed contamination can be caused by contaminated dust when it becomes airborne, or by carry-over contamination from the previous rearing.

Feed samples were not analysed in this study. It is however known that feed infection is associated with surface contamination (Friese et al., 2013; Maciorowski et al., 2007; Marin et al., 2011). Feed undergoes extensive treatments using chemicals (formic, hydrochloric, nitric, phosphoric, propionic, and sulfuric acids) and heating (between 70 and 90 °C) to control pathogen colonisation (Doyle & Erickson, 2006). In this study, 72 feed loaders and 120 feeders were swab-sampled before cleaning and after disinfection, over three consecutive cycles of the cleaning regime. The environmental swabs collected on feed loaders and feeders were analysed for the prevalence of *Salmonella* spp. and *Campylobacter* spp. through cross-tabulation.

It was anticipated that feed loaders would have low prevalence of *Salmonella* spp. and *Campylobacter* spp., because feed loaders are located at a higher location (about one meter from the floor); thus, birds had limited access to loaders. This expectation agrees with the results obtained in cycles 2 and 3 before cleaning, where the prevalence of *Salmonella* spp. was 8.3 % (1/12) for both cycles; whilst the prevalence of *Campylobacter* spp. was 8.3 % (1/12) and 16.7 % (2/12), respectively. However, in cycle 1 before cleaning, both *Salmonella* spp. and *Campylobacter* spp. had prevalence of 50 % (6/12) (Figure 5.4). The high bacterial prevalence observed in cycle 1 may be attributed to contaminated dust that settled in the feeders (Chinivasagam, Tran, Maddock, Gale,&

Blackall, 2009). After disinfection, *Salmonella* spp. was not detected in cycles 2 and 3, but were observed in cycle 1, with a prevalence of 16.7 % (2/12). Meanwhile, the prevalence of *Campylobacter* spp. was at 8.3 % (1/12), on all three cycles (Figure 5.4). Davies and Wray (1996) reported similar results, and concluded that pathogen re-contamination on disinfected feed loaders, may be caused by inefficient cleaning from the previous cleaning regime.

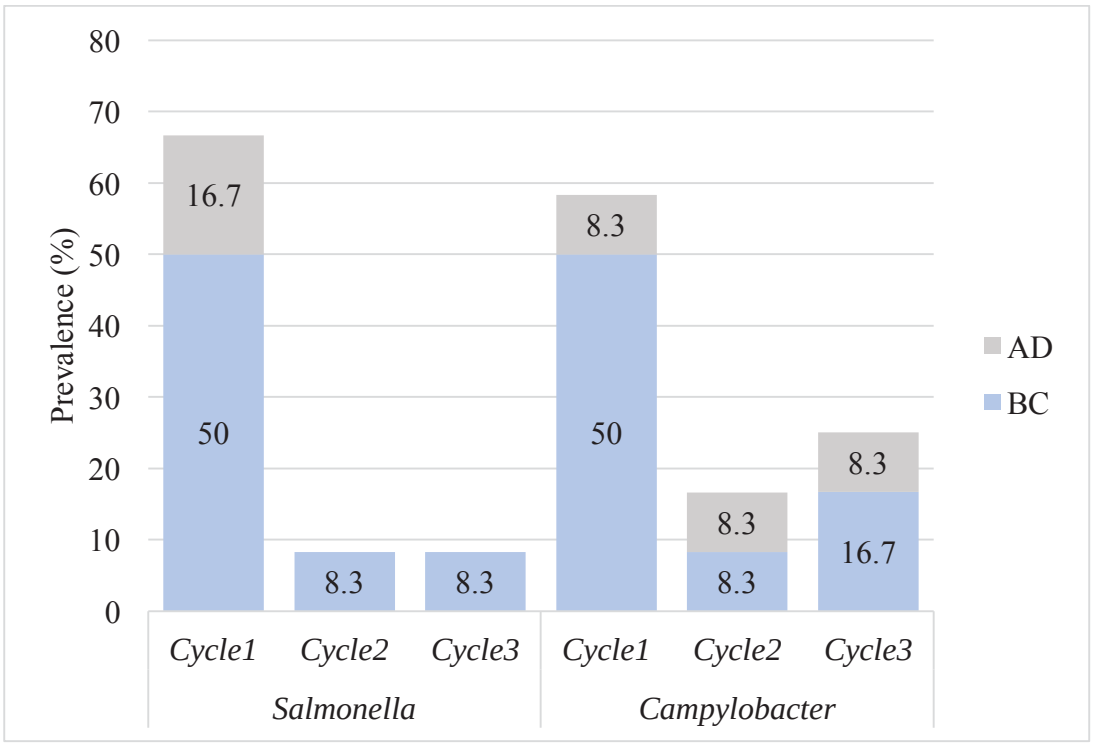

Figure 5.4 The prevalence of *Salmonella* and *Campylobacter* spp. in feed loaders during three consecutive cycles of the cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Feeders have higher chances of getting contaminated during rearing because birds get in contact with the equipment. The prevalence of *Salmonella* spp. was 35 % (7/20) in cycle 1 and 15 % (3/20) in cycle 2 before cleaning the shed. Whereas the prevalence of *Campylobacter* spp. was 40 % (8/20) and 20 % (4/20) in cycles 1 and 2 respectively

(Figure 5.5). The contamination on feeders during rearing was suspected to have been introduced by dusts, dirty litter, regurgitation of feed, or transfer of infection by beaks Herman et al., 2003; Marin et al., 2011). After disinfection, the prevalence of *Salmonella* spp. was 10 % (2/20) and 5 % (1/20) during cycle 1 and 2, while the prevalence of *Campylobacter* spp. was 10 % (2/20) in both cycles (Figure 5.5). The presence of both pathogens after disinfection is of concern, as this could lead to infection of birds. Residual feed left on feeders and/or disinfectants not properly applied were possible reasons for re-contamination. It was observed during sampling that feeders were not inverted to allow 'drip drying'. To prevent carry-over of infection to the next flock, it is important to dry shed equipment and shed surfaces (Allen & Newell, 2005).

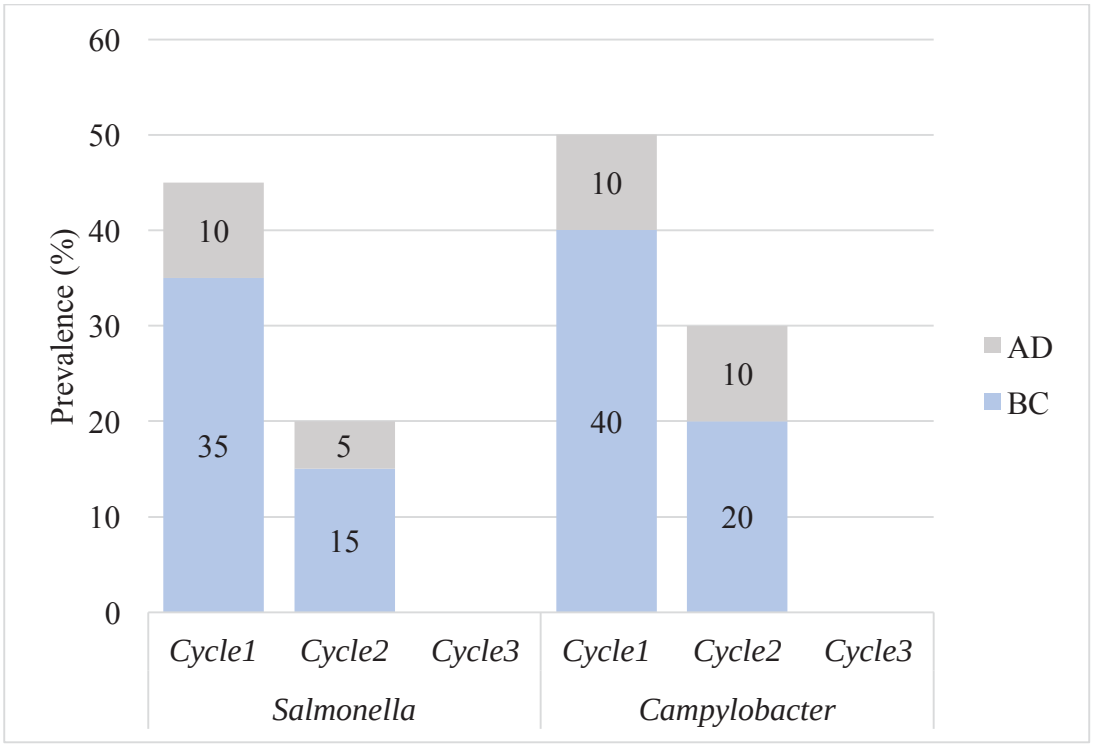

Figure 5.5 The prevalence of *Salmonella* and *Campylobacter* spp. in feeders during three consecutive cycles of the cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Samples collected on feed loaders (n = 72) and feeders (n = 120) also analysed for *S. aureus* and *E. coli* counts (Table 5.4 and 5.5). Before cleaning, mean counts of *S. aureus*

ranged from 6.75 to 4.84 Log_{10} CFU/mL, and 5.93 to 4.78 Log_{10} CFU/mL on feed loaders and feeders, respectively. Mean counts of *E. coli* were lower than those of *S. aureus,* which varied from 3.85 to 2.24 Log_{10} CFU/mL on feed loaders, and 4.83 to 1.82 Log_{10} CFU/mL on feeders. Comparing cell counts of *S. aureus* and *E. coli* before cleaning and after disinfection, mean counts of *S. aureus* decreased by 1.51 (cycle 1), 2.9 (cycle 2), and 2.48 (cycle 3) on feed loaders; and 2.15 (cycle 1), 2.01 (cycle 2), and 2.93 (cycle 3) on feeders, respectively. Meanwhile, *E. coli* was not detected in cycle 3 on both locations; but was reported in cycles 1 and 2, with mean decreases of 0.95 and 0.09 Log_{10} CFU/mL on feed loaders, and 2.45 and 1.5 Log_{10} CFU/mL on feeders, respectively. The decrease of *S. aureus* on feed loaders and feeders were higher compared to the decrease of *E. coli* after disinfection. This was unexpected because *E. coli* is a Gram –ve bacteria. *E. coli* has a thinner peptidoglycan layer compared to *S. aureus* (Gram +ve), sanitisers and disinfectants are expected to easily penetrate the cells causing them to shrivel and die (Mahalanabis et al., 2009). Moreover, the observations of *S. aureus* and *E. coli* having lower cell count decreases on feed loaders compared to feeders, may be explained by variability in cleaning standards of different teams. Feed loaders were expected to have a higher decrease in cell counts because they are constructed from metal. Generally, metal surfaces are easier to clean and disinfect due to their smoother surfaces (Poppe et al., 1991).

Table 5.4 Mean prevalence and counts of *S. aureus* and *E. coli* in samples collected in feed loaders

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	12 (100)	10 (83.3)	6.16 (0.91)	3.85 (1.09)
	AD	12 (100)	4 (33.3)	4.65 (0.98)	2.90 (0.81)
2	BC	9 (75)	9 (75)	6.75 (0.66)	2.24 (0.63)
	AD	1 (8.3)	1 (8.3)	3.85 (NA)	2.15 (NA)
3	BC	12 (100)	2 (16.7)	4.84 (0.51)	3.12 (1.02)
	AD	8 (66.7)	ND	2.36 (0.85)	ND

Note: ND = not detected; NA = not applicable; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Table 5.5 Mean prevalence and counts of *S. aureus* and *E. coli* in samples collected in feeders

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	18 (90)	17 (85)	5.77 (0.65)	4.83 (1.48)
	AD	18 (90)	2 (10)	3.62 (0.72)	2.38 (0.11)
2	BC	18 (90)	17 (85)	5.93 (1.11)	2.74 (1.29)
	AD	8 (40)	2 (10)	3.92 (0.93)	1.24 (0.34)
3	BC	19 (95)	8 (40)	4.78 (0.83)	1.82 (0.49)
	AD	12 (60)	ND	1.85 (0.48)	ND

Note: ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

5.2.5 Fan and vents

Fans and vents are important features of broiler sheds, as they manage the relative humidity and temperature during poultry production (Appleby et al., 2004; Aviagen, 2009; Broom & Fraser, 2015; Calvet et al., 2011; Mench et al., 2008). The equipment are also used for controlling build-up of ammonia gas from poultry waste to prevent incidences of respiratory diseases (Sonaiya & Swan, 2007).

Through the years, producers have installed modern ventilation systems. Large volumes of air are moved through the shed by a negative pressure, providing optimal conditions for broiler growth (Aviagen, 2009; Chinivasagam et al., 2009; Mench et al., 2008). Evidently, the large volume of air may contain a range of bacteria sourced from the external and/or internal environment of the shed. Therefore, proper disinfection of the fan ventilation system is vital to reduce chances of pathogens being airborne, especially when dust is present (Chinivasagam et al., 2009).

Ninety-six fans and 120 vents were swab sampled before cleaning and after disinfection, over three consecutive cycles of the cleaning regime. The collected swab samples (n = 96) on fans were analysed for the prevalence of *Salmonella* spp. and *Campylobacter* spp. through cross-tabulation (figure 5.6). Before cleaning, prevalence of *Salmonella* spp. on fans were 31.3 % (5/16), 6.3 % (1/16), and 18.8 % (3/16) in cycles 1, 2, and 3,

respectively. Compared to *Salmonella* spp., the prevalence of *Campylobacter* spp. before cleaning was higher with 37.5 % (6/16) observed in cycle 1, and 18.8 % (3/16) in cycles 2 and 3, respectively. After disinfection, *Salmonella* spp. was not detected in all three cycles, whilst the prevalence of *Campylobacter* spp. was only observed in cycle 2 at 12.5 % (2/16) (Figure 5.6). The efficiency of the cleaning regime applied on fans may be associated with the decrease of prevalence observed with *Salmonella* spp. and *Campylobacter* spp. However, since *Campylobacter* spp. was detected in cycle 2 after disinfection, it was suspected that cleaning teams operate in different standards.

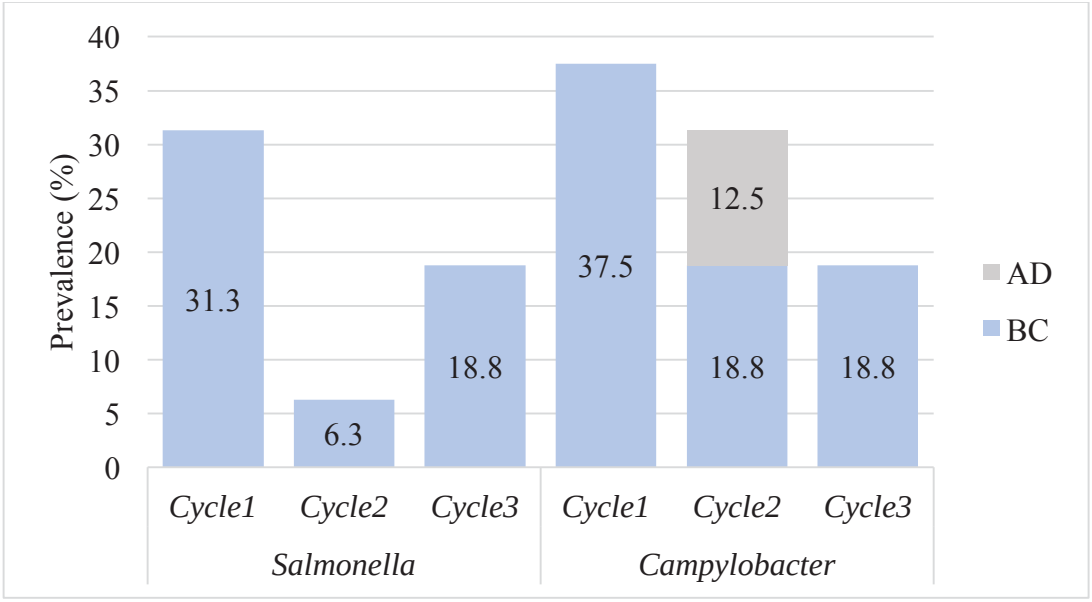

Figure 5.6 Prevalence of *Salmonella* and *Campylobacter* spp. in fans during three consecutive cycles of the cleaning regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Swab samples (n = 120) on vents were also analysed to determine the prevalence of *Salmonella* spp. and *Campylobacter* spp. through cross-tabulation (Figure 5.7). Both *Salmonella* spp. and *Campylobacter* spp. were not detected on vents in cycle 3 before cleaning and after disinfection. However, the prevalence of *Salmonella* spp. was observed at 50 % (10/20) and 15 % (3/20) in cycles 1 and 2 before cleaning the shed. Meanwhile, the prevalence of *Campylobacter* spp. was also 15 % (3/20) in cycle 2, but was detected to have a prevalence of 55 % (11/20) in cycle 1, respectively. After disinfection, the

prevalence of *Salmonella* spp. and *Campylobacter* spp. in cycle 2, were at 10 % (2/20). In cycle 1, the prevalence of *Salmonella* spp. and *Campylobacter* spp. were 25 % (5/20) and 30 % (6/20), respectively. The high prevalence after disinfection may be explained by the accumulated dusts observed to be trapped between the wires of the ventilation screen. Proper cleaning and disinfection on vents are important as they have been implicated as potential sources of contamination in broiler sheds (Chinivasagam et al., 2009). As air enters through the vents, it easily spreads contaminated dusts on feed and drinking water, thus potentially infecting flocks (Chinivasagam et al., 2009).

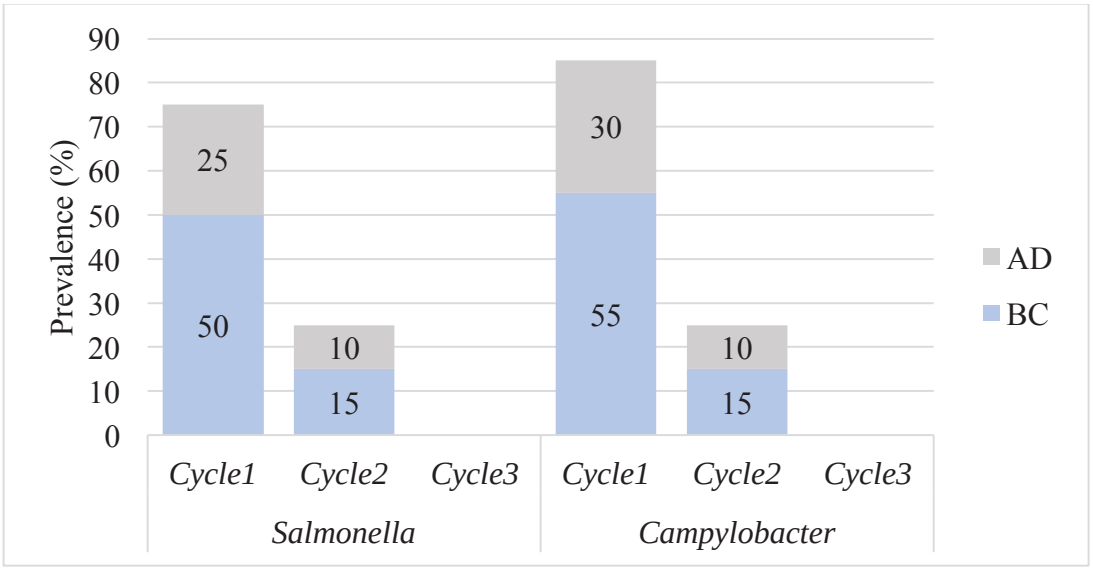

Figure 5.7 Prevalence of *Salmonella* and *Campylobacter* spp. in vents during three consecutive cycles of the cleaning and disinfection regimes

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

High bacterial prevalence of *Salmonella* spp. and *Campylobacter* spp. observed on the fan ventilation system in cycle 1 may be explained by seasonal variability in poultry production. In warmer climates, all fans are continuously operational, and vents are completely opened to control odour and moisture during rearing (Aviagen, 2009; Mench et al., 2008; Wabeck, 2002). In low temperature climates, vents are slightly opened, to reduce drought; as well as allowing air to mix with the temperature of the shed, before becoming in contact with birds (Aviagen, 2009; Mench et al., 2008; Vest & Tyson, 1991). Nevertheless, the increased exposure of contaminants from the external environment of

the farm, may partially explain the higher prevalence of both bacteria obtained in cycle 1. Previous studies also suggest that open ventilation during summer can encourage flies to act as vectors for pathogen leading to the spread of infection among flocks (McDowell et al., 2008; Newell & Fearnley, 2003; Vandeplas et al., 2010).

Table 5.6 Mean prevalence and counts of *S. aureus* and *E. coli* in samples collected in fans

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	16 (100)	13 (81.3)	5.77 (0.89)	3.93 (1.13)
	AD	15 (93.8)	4 (25)	3.77 (0.86)	2.49 (1.05)
2	BC	12 (75)	14 (87.5)	6.67 (0.79)	2.41 (0.68)
	AD	4 (25)	4 (25)	3.24 (1.00)	2.27 (0.67)
3	BC	14 (87.5)	14 (87.5)	5.6 (0.81)	1.89 (0.59)
	AD	13 (81.3)	ND	2.33 (0.58)	ND

Note: ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Table 5.7 Mean prevalence and counts of *S. aureus* and *E. coli* in samples collected in vents

Cycle	Stage of Cleaning	Prevalence (%)		Mean (SD) Log_{10} CFU/mL of positive samples	
		S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	BC	19 (95)	14 (70)	5.61 (0.80)	4.50 (1.10)
	AD	17 (85)	5 (25)	3.85 (1.31)	2.65 (0.95)
2	BC	17 (85)	10 (50)	5.92 (0.44)	2.45 (0.58)
	AD	7 (35)	2 (10)	3.73 (0.73)	1.48 (0.00)
3	BC	20 (100)	1 (5)	4.25 (1.02)	1.78 (NA)
	AD	17 (85)	ND	3.25 (1.04)	ND

Note: ND = not detected; NA = not applicable; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection

Samples obtained from fans (n = 96) and vents (n = 120) were also analysed to determine bacterial load of *S. aureus* and *E. coli* (Table 5.6 and 5.7). Before cleaning, mean counts of *S. aureus* ranged from 5.6 to 6.67 Log_{10} CFU/mL, and 5.92 to 4.25 Log_{10} CFU/mL on fans and vents, respectively. Whereas, mean counts of *E. coli* were lower than *S. aureus*, with counts ranging from 1.89 to 3.92 Log_{10} CFU/mL in fans and 1.78 to 4.50 Log_{10} CFU/mL in vents. The mean counts of *S. aureus* were not significantly different between cycles 1, 2, and 3; whilst the mean counts of *E. coli* were observed to have decreased by 1 Log_{10} between the cycles. The steady decrease of *E. coli* may be due to seasonal variability, especially because of the higher counts observed in cycle 1. Comparing counts before cleaning and after disinfection, the mean counts of *S. aureus* in cycle 1, 2, and 3 decreased by 2, 3.43, and 3.32 Log_{10} CFU/mL on fans; and 1.76, 2.19, and 1 Log_{10} CFU/mL on vents. *E. coli* was not detected in cycle 3 on fans and vents. However, in cycle 1 and 2, the mean counts decreased by 1.44 and 0.14 on fans, and 1.85 and 0.97 on vents respectively. The higher mean counts of *S. aureus* obtained after disinfection, compared to the mean counts of *E. coli* on fans and vents, indicated that *S. aureus* may be less sensitive to the disinfectant used in this study.

5.3 Bacterial enumeration using multiplex qPCR

Microbiological assays are still used to regularly monitor potential contamination trends and microbial risks (Rodríguez-Lázaro, 2013). However, qPCR has become an important quantification tool due to its ability to rapidly detect segments of the DNA that are unique to the species and strain level (WHO, 2003). For instance, qPCR methods are now used to detect and quantify *E. coli* O157 in raw milk to support investigations in food safety (Paul, Van Hekken, & Brewster, 2013), *Campylobacter* spp. in chicken rinse for assessing initial contamination at slaughterhouses (Botteldoorn et al., 2008), as well as detecting and enumerating pathogenic *Candida* cells to evaluate water safety (Brinkman et al., 2003).

5.3.1 PCR optimisation

The primer and probe sequences used in this study for detecting and quantifying foodborne pathogens, was developed and validated by Cremonesi et al. (2014). This study adopted similar reaction conditions (50 ºC for 2 min, 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 60 s) for amplifying DNA targets in a singleplex qPCR assay. TaqMan® Environmental Master Mix, which contains essential reaction buffers (Taq DNA polymerase, dNTPs, $MgCl_2$, KCl, and stabilisers), was also adopted for efficient amplification of target DNA. Optimal primer and probe concentrations of 0.9-µm and 0.25-µm for all targets were also replicated. The same parameters of the qPCR reaction conditions were also carried out, except that TaqMan® Environmental Master Mix which was replaced with TaqMan® Gene Expression Master Mix. This is due to the manufacturer's recommendation for optimal use in multiplex studies. When the DNA amplification of *Salmonella* spp. with the above conditions was implemented, the results showed non-specific amplification, as demonstrated in Figure 5.8. Since the composition of both master mixes were different, optimisation was vital to generate good amplification curves.

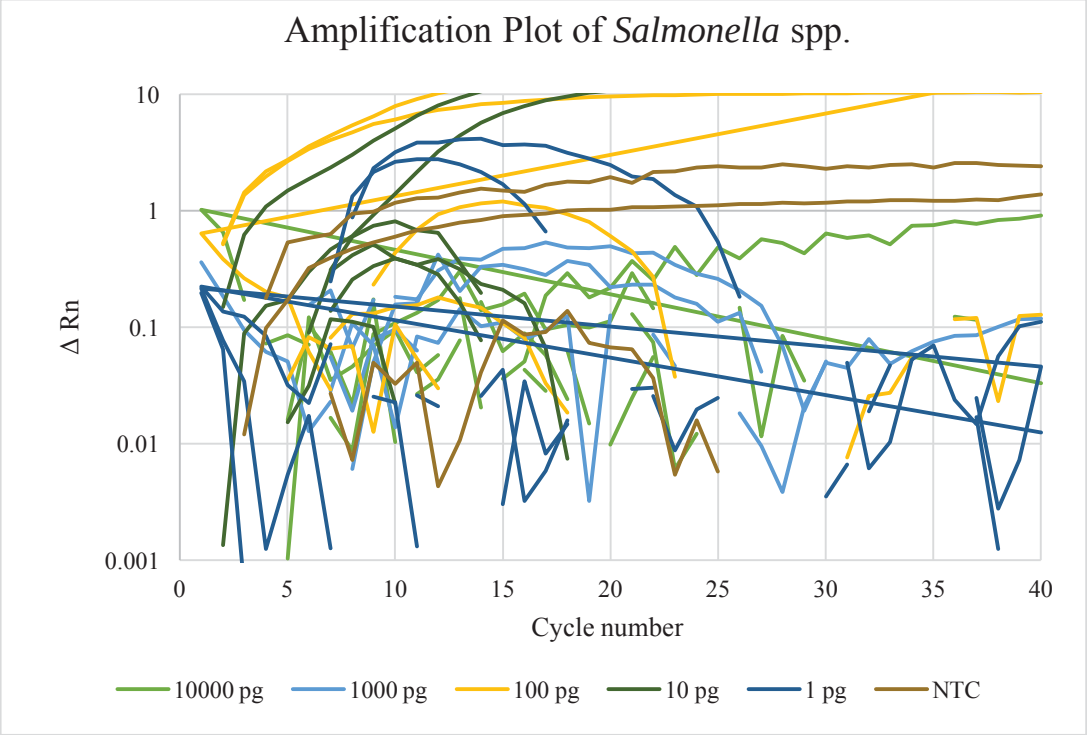

Figure 5.8 *Salmonella* spp. qPCR amplification plot (10000-pg to one-pg)
Note: NTC = No template control (Negative control - Milli-Q water); n = triplicates

To optimise reaction conditions in a single PCR run, gradient PCR analysis was performed. This study evaluated annealing temperatures between 60 – 52 ºC, and primer concentrations of 0.9-µM, 0.5-µM and 0.3-µM. Standardising reaction conditions is important for multiplex assays, to amplify specific DNA products with equal efficiency and sensitivity, as well as minimising adverse primer interactions (Sint et al., 2012). Thus, selecting the optimal PCR conditions depends entirely on the intensity and clarity of the bands generated by gel electrophoresis (Sint et al., 2012).

The temperature gradient PCR of *Salmonella* spp., *E. coli* O157:H7 and *S. aureus* at 0.9-µM primer concentration (Figure 5.9) yielded low DNA products as demonstrated by the faint bands observed in gel electrophoresis. However, during temperature gradient, DNA amplification of *E. coli* O157:H7, non-specific binding was observed at 60 - 58ºC. This phenomenon usually occurs when primers non-specifically bind to the template, producing bands that are either bigger or smaller than the target band.

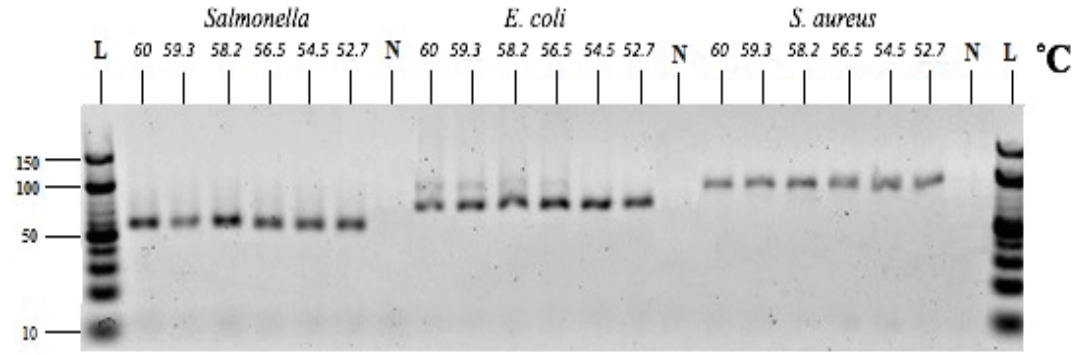

Figure 5.9 Temperature gradient PCR of *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* at 0.9-µM primer concentration
Note: Lane L = 10-bp DNA ladder; N = Negative control (Milli-Q water)

The intensity of the bands was brighter during temperature gradient PCR of *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, at 0.5-µM primer concentration (Figure 5.10). Similarly, non-specific binding was still observed during *E. coli* O157:H7 DNA amplification reactions at 60 – 58 ºC. The non-specific bands identified above the *E. coli* O157:H7 targets, became more pronounced when the primer concentration decreased to 0.5-µM.

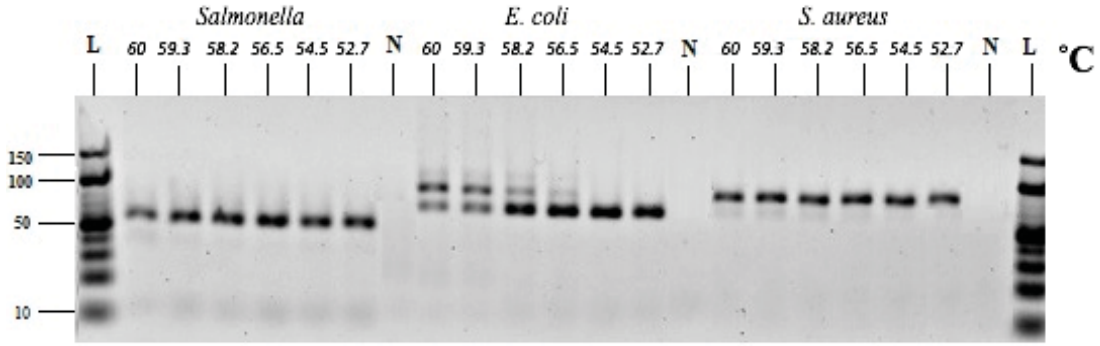

Figure 5.10 Temperature gradient PCR of Salmonella spp., E. coli O157:H7, and S. aureus at 0.5-µM primer concentration
Note: Lane L = 10-bp DNA ladder; N = Negative control (Milli-Q water)

Meanwhile, *Campylobacter* spp. DNA amplification produced comparable high DNA yield at 0.5-µM and 0.9-µM primer concentration as illustrated by the intensity of the bands in Figure 5.11.

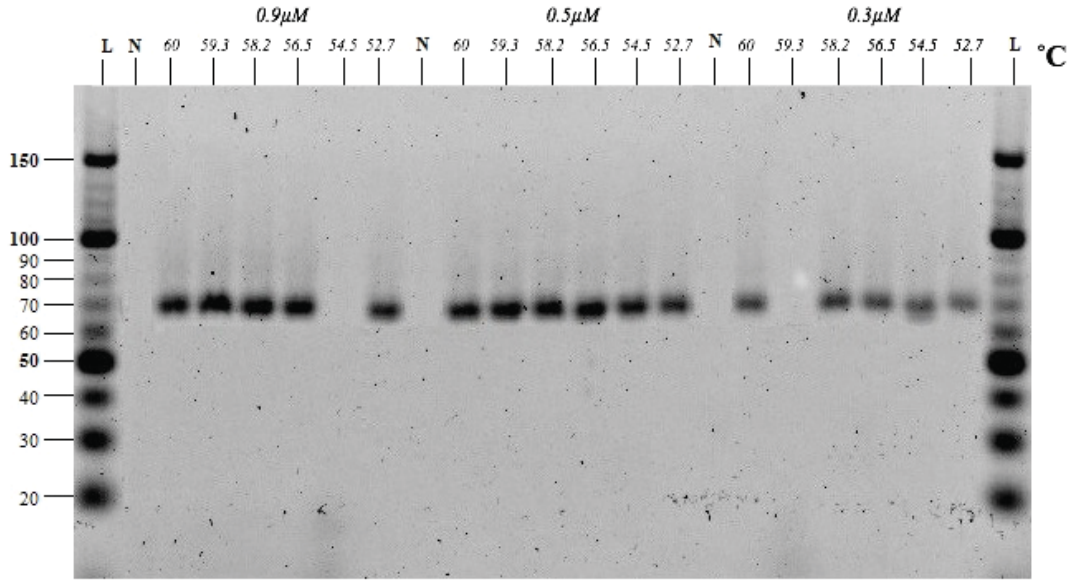

Figure 5.11 Temperature gradient PCR of *Campylobacter* spp. at 0.9-µM, 0.5-µM, and 0.3-µM primer concentration

Note: Lane L = 10-bp DNA ladder; N = Negative control (Milli-Q water). Lane 7 (0.9-µM at 54.5 ºC) and lane 18 (0.3-µM at 59.3 ºC) were blank due to evaporation caused by opening PCR tubes during the experiment

Temperature gradient PCR was also performed at 0.3-µM, to investigate whether the DNA amplification of each target was more specific at a lower primer concentration. In this case, the DNA amplification reaction of *S. aureus* ceased as displayed by the faint bands observed in Figure 5.12. Similarly, the DNA yield of *Campylobacter* spp. amplification at 0.3-µM decreased as shown by the bands in Figure 5.11. Temperature gradient of *Salmonella* spp. and *E. coli* O157:H7at 0.3-µM (Figure 5.12) lead to non-specific binding and DNA smearing. The smeared DNA bands could be due to a variety of factors, including DNA degradation and over expression of target DNA. The low molecular bands observed in *Salmonella* spp. DNA amplification could be due to primer dimers (low DNA bands). Primer dimers manifest when primer pairs hybridise due to the complementary bases of primers.

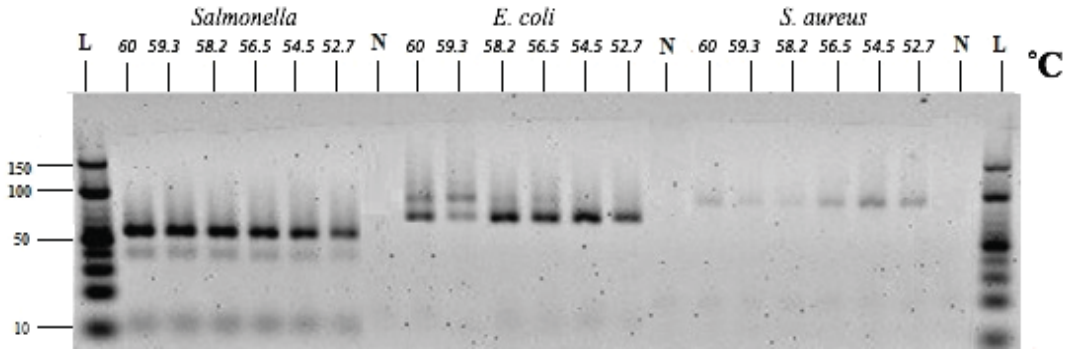

Figure 5.12 Temperature gradient PCR of *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* at 0.3-µM primer concentration
Note: Lane L = 10-bp DNA ladder; N = Negative control (Milli-Q water)

Gradient PCR results demonstrate the optimal reaction conditions that can amplify all bacteria targets in a multiplex PCR (Lorenz, 2011; Sint et al., 2012). The primer concentration of 0.5-µm yielded the most distinct bands for all targets, compared with 0.9-µm or 0.3-µm. Moreover, to standardise the reaction conditions of this study, the optimal annealing temperature was based on the DNA amplification of *E. coli* O157:H7. *E. coli* O157:H7 was observed to cause non-specific binding when the annealing temperature increased to 58 – 60 ºC range.

The annealing temperature of 57 ºC was the most suitable for this study. Typically, increasing the annealing temperature enhanced the DNA amplification specificity, which reduced primer dimers and non-specific binding (Lorenz, 2011). However, in this case, the results showed the complete opposite, whereby DNA amplification specificity was reduced when the annealing temperature increased. Nevertheless, since the optimal annealing temperature of each assay depends on various factors (nucleotide sequence, DNA length, and primer concentrations) (Arya et al., 2014; Markoulatos, Siafakas, & Moncany, 2002), the annealing temperature of 57 ºC was chosen for all targets based on the gradient PCR results (Figure 5.9- 5.12).

5.3.2 Verifying optimised reaction conditions through standard curve generation of singleplex qPCR assay

Absolute quantification using the standard curve method are used in food and environmental microbiology research (Brankatschk et al., 2012). It is based on the comparison of cycle threshold (C_t) values with a standard curve generated by known concentration of target DNA. The DNA standards used in this study were extracted from bacterial cells that had primer binding sites identical to those found in sequences of the experimental target (Fraga et al., 2008). Once extracted, the DNA standard was serially diluted in 10-fold increments. Each dilution was run in triplicate and the average C_t was plotted against the absolute amount to generate the standard curve. Comparing standard C_t and sample C_t, provided the estimated amount of the target present in a sample.

Not only does the standard curve estimate the target quantity of a sample, but it also assesses the efficiency of the qPCR assay. The amplification efficiency was calculated from the equation E = [10(-1/slope) – 1], where a slope of -3.32 is equals to 100 % efficiency. An efficiency of 100 % represented perfect doubling of DNA in every cycle (Pestana, Belak, Diallo, Crowther, & Viljoen, 2010). However, achieving this is not always possible because of various factors such as; secondary structure formation in DNA template, contamination in DNA template preparation, or poor qPCR reaction conditions (Bustin et al., 2009; Fraga et al., 2008). Thus, the MIQE (Minimum Information Quantitative Experiments) guideline recommends that the efficiency of the reaction should be as close to100% (Bustin et al., 2009).

The range of acceptable DNA amplification efficiency varies between studies. For instance, Gordillo et al. (2014) accepted amplification efficiency between 80 to 110 % when multiplex qPCR method was developed for quantifying *E. coli* O157:H7 *fliC* and *rfbE* genes in meat products. Whilst an amplification efficiency between 90 to 100% was deemed acceptable by Al-Tebrineh et al. (2012), when they developed qPCR assays for detecting and quantifying toxic genes of cyanobacteria in environmental samples.

In this study, the amplification efficiency between 80 to 115 % was acceptable for quantifying environmental samples, in agreement with Zhang and Fang (2006), who evaluated applications of qPCR assays from various studies. Zhang and Fang (2006) determined that standard curves should have a slope between –3.9 and –3.0, which corresponded to an amplification efficiency of 80 to 115 %.

PCR efficiency is closely related to the specificity of the assay. At low specificity, the efficiency low (> 80 %) due to inevitable errors during serial dilutions, or reagents amplifying non-specific products (primer dimers) (Van Pelt-Verkuil et al., 2008). Low specificity also occurs when an increase of DNA amplification (< 115 %) is observed due to assay inhibitors. High assay inhibitors are usually found in DNA standards with high template concentrations. This then causes delayed C_t readings, thus affecting the efficiency of the assay. Meanwhile, samples with lower template concentrations have lower levels of inhibitors, therefore, C_t readings are minimally delayed (Life-Technologies, 2014; Logan, Edwards, & Saunders, 2009).

Another critical parameter of the standard curve when evaluating qPCR reactions is the correlation coefficient (R^2). When performing linear regression analysis, R^2 measures the linearity of the qPCR reaction. The R^2 measures replicate reproducibility of an assay when generating standard curves, and thus maintains data accuracy for the samples (Life-Technologies, 2014). The R^2 value of each target should be > 0.98 (Broeders et al., 2014). A low R^2 (< 0.98) value, suggests errors associated with loading standards into the reaction mixture, or serial dilution of standards.

The optimised reaction conditions from the PCR optimisation experiment were verified using singleplex qPCR. To determine the linearity of the assay, standard curves were generated by performing 10-fold serial dilution of 50-ng/µl DNA stock. Two-µl of each dilution were used as a template, which covered the range of one to 10,000-pg per reaction. *Salmonella* spp., *Campylobacter* spp., *S. aureus*, and *E. coli* O157:H7 DNA amplification showed linear relationship between gDNA input (pg) and C_t values, with an R^2 of 0.99. Whilst the efficiency for simultaneous detection of *Salmonella* spp.,

Campylobacter spp., *S. aureus*, and *E. coli* O157:H7 yielded an average efficiency of 100.77 %, 93.42 %, 93.26 %, and 91.13 % respectively (Figure 5.12).

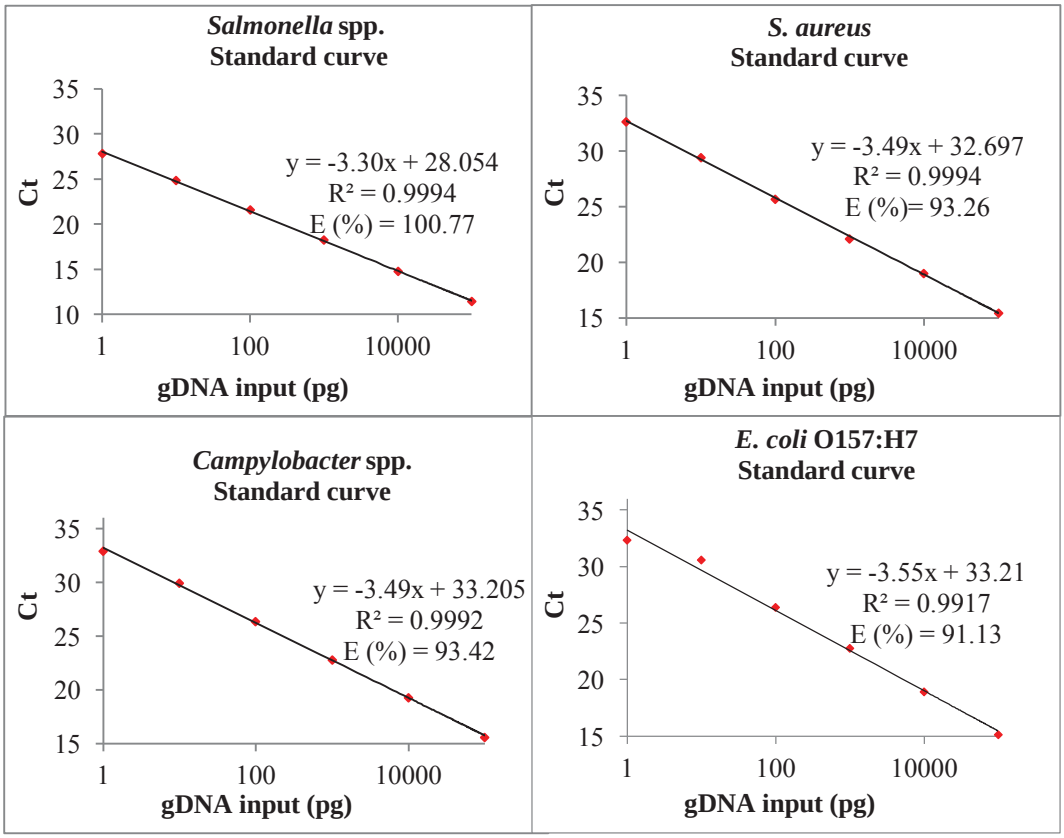

Figure 5.13 Standard curves for *Campylobacter* spp., *Salmonella* spp., *S. aureus*, and *E. coli* 0157:H7 singleplex assays

Note: Serial dilutions of each DNA standard ranged from one to 10,000-pg per reaction. All standard curves are average results from independent triplicate data sets. Efficiency (E) was calculated based on the slope determined by linear regression analysis

5.3.3 Optimisation of multiplex qPCR assay

Multiplex qPCR allows the detection of different genera or species present in the same environmental matrices (Al-Tebrineh et al., 2012). Many real-time thermocyclers can amplify more than one target DNA in a single reaction, because of the development of hydrolysis probes (Kubista et al., 2006). Each target in a multiplex reaction has a specific primer pair and labelled probe. The probe has a unique fluorescent dye that fluoresce at different wavelengths, allowing separate quantification of each target (Eckford-Soper &

Daugbjerg, 2015). Because of the increase in sample throughput, multiplex qPCR assays reduce labour and cost (Al-Tebrineh et al., 2012; Hyeon, Park, Choi, Holt, & Seo, 2010).

Several studies have reported efficient use of multiplex PCR systems in environmental and food matrices. Park et al. (2011) designed a multiplex qPCR assay that detects and quantifies *Campylobacter* spp., *Salmonella* spp. and *E. coli* O157:H7, in watershed samples. *S.* Typhimurium and *E. coli* O157:H7 standard curves were generated by calculating DNA copy numbers, and performing 10-fold DNA dilutions that ranged from 7.11×10^7 to 7.11×10^1 copies/µl. *C. jejuni* standard curve ranged between 7.11×10^7 and 7.11×10^0 copies/µl due to the high sensitivity of the primer pair. Josefsen, Jacobsen, and Hoorfar (2004) also designed a multiplex qPCR assay to detect and quantify thermotolerant *Campylobacter* spp. in chicken carcases, by targeting the 16S rRNA gene. qPCR estimates (CFU/mL) were quantified using standard curves generated from 10-fold bacterial cell dilutions that ranged between 10^2 and 10^7 CFU/mL, after a 20-hour pre-enrichment.

Multiplex qPCR standard curves based on DNA concentration (pg) was used to assess the efficiency of the assay in this study. The DNA standard curve was constructed by performing 10-fold dilutions of 50-ng/µl DNA stock. The representative dilution of each target (*Salmonella* spp. - *S. aureus* / *Campylobacter* spp. - *E. coli* 0157:H7) was combined, and four-µl of each dilution were used as a template, covering the range of one to 10,000-pg per reaction.

To investigate assay efficiencies between targets during multiplex qPCR, singleplex assays were run in parallel. Standard curves generated from *Salmonella* spp. and *S. aureus* DNA targets, showed linear relationship between pg and C_t, with an R^2 of 0.99 (Figure 5.14). *Salmonella* spp. and *S. aureus* DNA amplification demonstrated no competition during multiplex qPCR. The assay efficiency of *Salmonella* spp. and *S. aureus* in singleplex and multiplex assays were comparable (> 5 %). The DNA amplification efficiency of *Salmonella* spp. in singleplex and multiplex assays were observed to be 99.92 % and 101.46 %; Whilst the efficiency of *S. aureus* were 87.95 % and 87.60 % in singleplex and multiplex assays respectively (Figure 5.14).

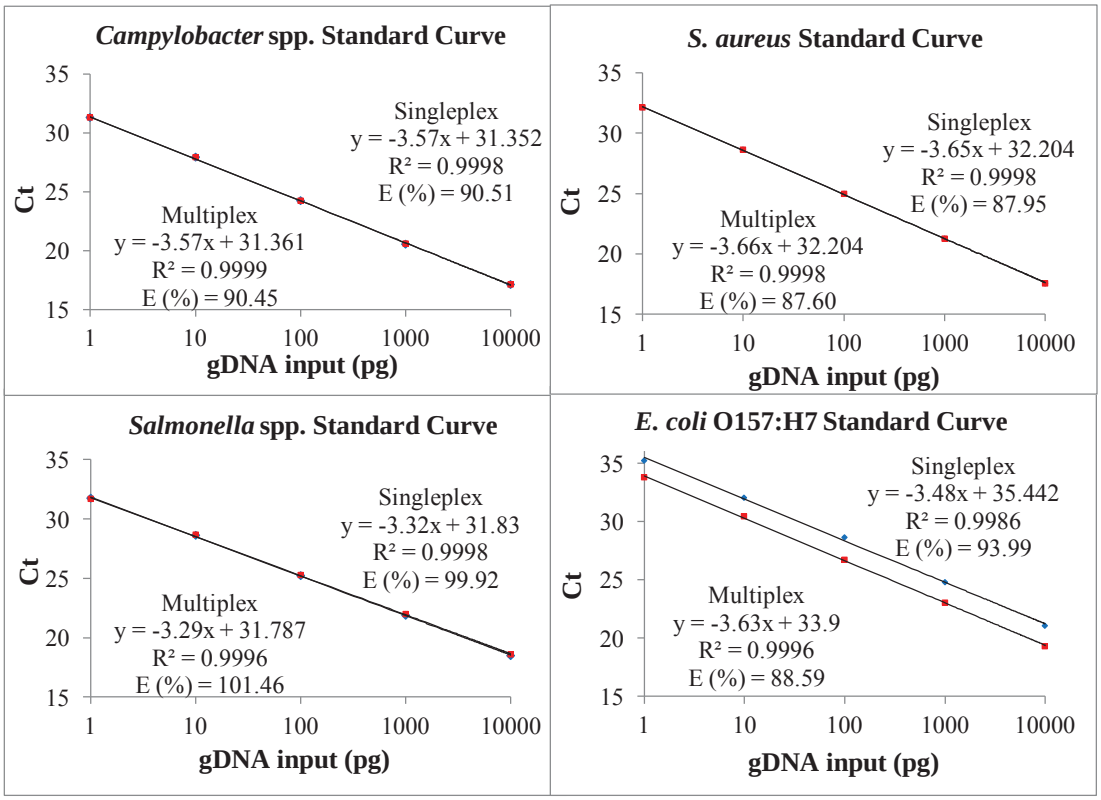

Figure 5.14 Standard curves for *Campylobacter* spp., *Salmonella* spp., *S. aureus*, and *E. coli* O157:H7 singleplex (red) and multiplex (blue) assay

Note: Serial dilutions of each DNA standard ranged from one to 10,000-pg per reaction. All standard curves are the average results from independent triplicate data sets. Efficiency (E) was calculated based on the slope determined by linear regression analysis

Meanwhile, *Campylobacter* spp. and *E. coli* O157:H7 multiplex assays demonstrated competition during co-amplification of each DNA target. The DNA amplification efficiency of *Campylobacter* spp. in singleplex and multiplex assays were 90.51 % and 90.45 %; whereas the amplification efficiencies of *E. coli* O157:H7 were 93.99 % and 88.59 % in singleplex and multiplex assays, respectively (Figure 5.14). The differences between the DNA amplification efficiency of *E. coli* O157:H7 singleplex and multiplex assays were more than 5 %, where a reduction of efficiency was observed. This suggested that the DNA amplification of *Campylobacter* spp. suppressed the DNA amplification of *E. coli* O157:H7 because *Campylobacter* spp. was observed to cross the threshold baseline earlier in the multiplex reaction (Table 5.8).

Table 5.8 Sensitivity of singleplex and multiplex assay

	Assay	C_t (threshold cycle)				
		DNA Concentration (pg)				
		10000	1000	100	10	1
C	Singleplex	17.11±0.05	20.53±0.10	24.21±0.06	27.90±0.07	31.29±0.02
	Multiplex	17.07±0.02	20.60±0.01	24.20±0.02	27.89±0.01	31.30±0.03
S	Singleplex	18.49±0.05	21.85±0.03	25.23±0.01	28.61±0.01	31.73±0.03
	Multiplex	18.56±0.01	21.95±0.01	25.26±0.06	28.64±0.04	31.65±0.04
SA	Singleplex	17.57±0.05	21.24±0.02	24.97±0.05	28.64±0.06	32.11±0.24
	Multiplex	17.62±0.04	21.47±0.03	24.97±0.01	28.70±0.04	32.31±0.06
E	Singleplex	21.06±0.04	24.76±0.06	28.60±0.11	32.03±0.14	35.20±0.18
	Multiplex	19.30±0.02	23.02±0.06	26.72±0.05	30.42±0.04	33.75±0.14

Note: Average ± SD are based on triplicate analysis of *Campylobacter* spp. (C), *Salmonella* spp. (S), *S. aureus* (SA) and *E. coli* 0157:H7 (E) DNA amplification

The main objective of multiplex qPCR is to accurately quantify multiple targets in one reaction, without competition from non-target DNA or inhibiting chemical compounds (Eckford-Soper & Daugbjerg, 2015). Therefore, to achieve high assay efficiency, limiting primer concentrations or increasing the concentration of other components is vital. In this study, primer-limiting conditions were utilised, whilst maintaining master mix reagents and cycling conditions of the optimised singleplex method. The primer-limiting experiment was carried out in singleplex and multiplex qPCR (in parallel) until equivalent C_t values were observed (> 1 C_t) (Al-Tebrineh et al., 2012). As the DNA amplification of *Campylobacter* spp. supressed the DNA amplification of *E. coli* O157:H7 in multiplex qPCR, the primer concentration of *Campylobacter* spp. was adjusted whilst maintaining the primer concentration of *E. coli* O157:H7. When *Campylobacter* spp. primer concentration was lowered to 0.45-µM and 0.40-µM, the C_t difference of *E. coli* O157:H7 singleplex and multiplex assay was more than 1 C_t (Table 5.9). This indicated that in multiplex qPCR, *Campylobacter* spp. DNA amplification still supressed the DNA amplification of *E. coli* O157:H7.

Table 5.9 Multiplex optimisation 1

			C_t (threshold cycle)		
			10000-pg	100-pg	1-pg
Singleplex	E	0.50-µM	17.55	24.65	32.30
	C	0.45-µM	17.43	24.49	31.00
		0.40-µM	17.47	24.54	31.62
Multiplex	E	0.50-µM	15.89	23.25	30.34
		0.50-µM	15.81	22.90	30.44
	C	0.45-µM	17.65	24.66	31.66
		0.40-µM	17.56	24.43	31.58
C_t Difference	E	0.50-µM	1.66	1.40	1.96
		0.50-µM	1.74	1.75	1.86
	C	0.45-µM	-0.22	-0.17	-0.66
		0.40-µM	-0.09	0.11	0.04

Note: C_t values presented are based on triplicates of *E. coli* 0157:H7 (E) and *Campylobacter* spp. (C) DNA amplification

Further optimisation of primers was performed by reducing *Campylobacter* spp. primer concentration to 0.35-µM – 0.15-µM. The consistency of C_t values (> 1 C_t) from primer-limiting *Campylobacter* spp., indicated that the primer concentration of 0.15-µM could be used to produce reliable results in duplex reactions (Table 5.10; Optimisation Trial 2). However, the C_t difference for *E. coli* O157:H7 DNA amplification in both singleplex and multiplex assay was still over 1 C_t, where 1.27 – 1.79 was observed (Table 5.10; Optimisation Trial 2).

Reducing *Campylobacter* spp. primer concentrations did not produce equivalent C_t values (> 1 C_t) when *E. coli* O157:H7 DNA was amplified in singleplex and multiplex assays (Table 5.10; Optimisation Trial 2). Therefore, primer-limiting *E. coli* O157:H7 (0.30-µM to 0.85-µM) was performed to observe changes in C_t values, especially when *Campylobacter* spp. was already primer-limited to 0.15-µM. Table 5.10 (Optimisation Trial 3) shows that primer-limiting *E. coli* O157:H7 did not produce desirable results, because a C_t difference of 1.5 – 1.81 was observed between singleplex and multiplex assay.

Table 5.10 Multiplex optimisations 2 and 3

Optimisation Trial 2								
			C_t (threshold cycle)				C_t (threshold cycle)	
			10000-pg	100-pg			10000-pg	100-pg
Singleplex	E	0.50-µM	22.27	29.48	C	0.15-µM	19.62	26.86
	C	0.35-µM	14.53	21.37	E	0.30-µM	22.11	29.6
		0.30-µM	14.75	21.22		0.40-µM	22.31	29.59
		0.25-µM	14.26	22.32		0.60-µM	21.97	29.65
		0.20-µM	14.82	21.63		0.70-µM	22.31	29.94
		0.15-µM	14.47	21.42		0.80-µM	22.61	30.15
Multiplex	E	0.50-µM	20.59	27.96	C	0.15-µM	19.62	26.87
		0.50-µM	20.52	27.88		0.15-µM	19.76	27.07
		0.50-µM	20.48	27.68		0.15-µM	19.65	26.96
		0.50-µM	20.74	28.12		0.15-µM	19.74	26.89
		0.50-µM	20.93	28.21		0.15-µM	19.74	26.88
	C	0.35-µM	14.37	21.66	E	0.30-µM	20.55	27.79
		0.30-µM	14.36	21.45		0.40-µM	20.59	28.09
		0.25-µM	14.4	21.63		0.60-µM	20.5	27.99
		0.20-µM	14.61	21.77		0.70-µM	20.9	28.3
		0.15-µM	14.48	21.83		0.80-µM	20.96	28.37
C_t Difference	E	0.50-µM	1.68	1.52	C	0.15-µM	0	-0.01
		0.50-µM	1.75	1.6		0.15-µM	-0.14	-0.21
		0.50-µM	1.79	1.8		0.15-µM	-0.03	-0.1
		0.50-µM	1.53	1.36		0.15-µM	-0.12	-0.03
		0.50-µM	1.34	1.27		0.15-µM	-0.12	-0.02
	C	0.35-µM	0.16	-0.29	E	0.30-µM	1.56	1.81
		0.30-µM	0.39	-0.23		0.40-µM	1.72	1.5
		0.25-µM	-0.14	0.69		0.60-µM	1.47	1.66
		0.20-µM	0.21	-0.14		0.70-µM	1.41	1.64
		0.15-µM	-0.01	-0.41		0.80-µM	1.65	1.78

Note: C_t values presented is based on triplicates of *E. coli* 0157:H7 (E) and *Campylobacter* spp. (C) DNA amplification

Primer-limiting Campylobacter spp. and *E. coli* O157:H7 targets did not produce equivalent Ct values during singleplex and multiplex assays. This suggested that further optimisation was required by altering the composition of the master mix used. Despite the manufacturer's recommendation on TaqMan® Gene Expression Master Mix for multiplex studies, it does not have optimal reagent conditions for amplifying *Campylobacter* spp. and *E. coli* O157:H7 target genes. The competition between the two targets were most likely due to reaction components (DNA polymerase, nucleotides, and buffer composition, including magnesium) becoming limited in later cycles (Eckford-

Soper & Daugbjerg, 2015). This problem may be achieved by making custom made master mix designed specifically for the DNA targets being amplified (Eckford-Soper & Daugbjerg, 2015). For instance, Al-Tebrineh et al. (2012) reported that the presence of accumulated products in the later cycles inhibited DNA polymerase activity. The addition of DNA polymerase in the reaction mixture reduced inhibition, and supported multiplex amplification (Al-Tebrineh et al., 2012; Kainz, 2000). This type of optimisation was not done in this study due to budget and time constraints. Therefore, *Campylobacter* spp. and *Salmonella* spp. multiplex reactions were validated, and *S. aureus* was used as basis to confirm that the qPCR method can be used for CFU/mL determination.

5.3.4 *Salmonella* spp. and *Campylobacter* spp. multiplex qPCR reaction

Salmonella spp. and *Campylobacter* spp. singleplex and multiplex assays were performed to determine whether C_t values were comparable, and assay efficiencies were optimal. To standardise reaction conditions, primer concentration used in the multiplex reaction was 0.5-µM for both DNA targets. The data demonstrated comparable C_t values with 0.11 - 0.45 difference for *Salmonella* spp., and 0.08 - 0.46 difference for *Campylobacter* spp. (Table 5.11). The assay efficiencies of *Salmonella* spp. and *Campylobacter* spp. DNA amplifications were also within limits (80 to 115 %) and an R^2 of > 0.98 (Figure 5.15).

Table 5.11 Singleplex and multiplex reactions of *Salmonella* spp. and *Campylobacter* spp.

		C_t (threshold cycle)				
		DNA Concentration (pg)				
	Assay	10000	1000	100	10	1
C	Singleplex	19.40±0.15	23.74±0.25	27.19±0.02	30.05±0.26	32.92±.0.37
	Multiplex	19.91±0.25	23.39±0.17	26.87±0.04	30.51±0.03	33.00±0.55
S	Singleplex	18.56±0.03	21.88±0.06	25.23±0.02	28.59±-0.1	31.74±0.13
	Multiplex	18.67±0.25	22.22±0.05	25.45±0.05	28.73±0.08	31.29±0.52

Note: Average ± SD of C_t is based on triplicate results of *Salmonella* spp. (S) and *Campylobacter* spp. (C) DNA amplification

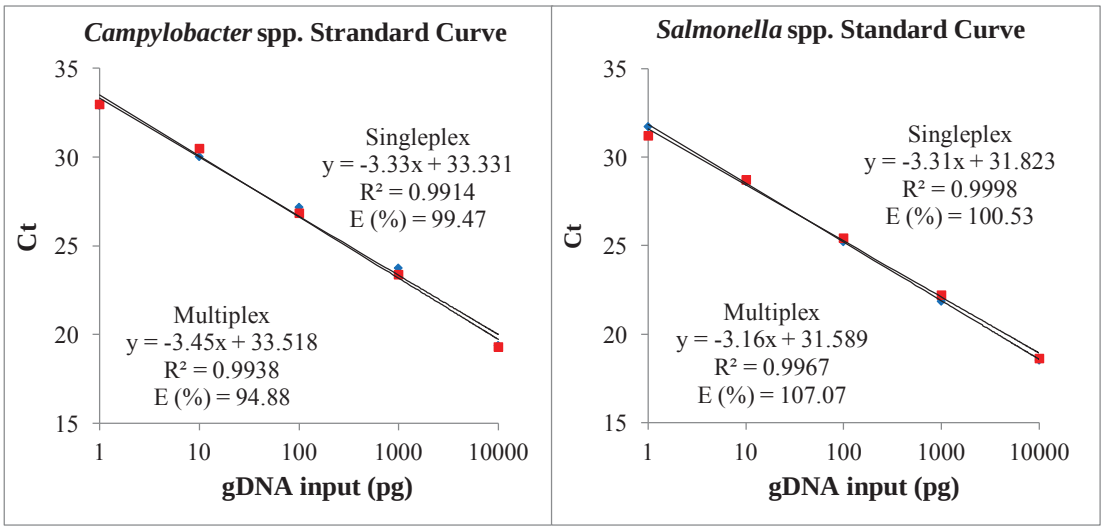

Figure 5.15 Standard curves for *Campylobacter* spp. and *Salmonella* spp. singleplex (red) and multiplex (blue) assays
Note: Serial dilutions of each DNA standard ranged from one to 10,000-pg per reaction. All standard curves were average results of independent triplicate data sets. Efficiency (E) was calculated based on the slope determined by linear regression analysis

5.3.4.1 Effects of different amounts of target pathogen on the sensitivity of multiplex qPCR

Bacterial pathogens can co-exist at different concentrations in the sample (Van Giau, Nguyen, Nguyen, Le, & Nguyen, 2016). Therefore, the ability of multiplex qPCR assay to amplify multiple DNA targets in different concentrations should be evaluated. To assess the sensitivity of the multiplex qPCR, a mixture of different bacterial concentrations was co-amplified, where one target was kept consistent (10000-pg) and the other target varied in DNA concentration (from one to 10,000-pg) (Eckford-Soper & Daugbjerg, 2015).

Table 5.12 shows no assay inhibition when *Campylobacter* spp. and *Salmonella* spp. DNA targets were amplified, especially when *Campylobacter* spp. was present at high DNA concentrations (10,000-pg), and *Salmonella* spp. was present in varying DNA concentrations (one to 10,000-pg). However, when *Salmonella* spp. was present at high DNA concentration (10,000-pg), and *Campylobacter* spp. had varying DNA

concentrations (one to 10,000-pg), the DNA amplification of *Campylobacter* spp. weakened from one to 100-pg. Previous studies have reported similar results, where the detection of a target was inhibited when the other target was present at high concentrations (Dai, Peng, Chen, Cheng, & Wu, 2013; Hyeon et al., 2010; Wang, Li, & Mustapha, 2007). They also reported that the detection of target pathogens was not influenced when one target was present in moderate or low concentrations (Dai et al., 2013; Hyeon et al., 2010; Wang et al., 2007). This indicates that high concentration of one target gene interferes with the DNA amplification of the other target gene at low concentration. This leads to increased C_t values, and results to a decrease in assay sensitivity (Dai et al., 2013).

Table 5.12 Evaluating the sensitivity of duplex reactions based on different bacterial concentration

		Singleplex	Multiplex	C_t Difference	SD of Ct Difference
C	10,000-pg	19.44	19.39	0.05	0.03
S	10,000-pg	19.98	20.00	-0.02	0.02
	1,000-pg	23.57	23.47	0.10	0.07
	100-pg	27.16	26.88	0.28	0.19
	10-pg	30.35	30.30	0.05	0.03
	1-pg	33.78	33.63	0.15	0.11
S	10,000-pg	19.98	20.02	0.32	0.03
C	10,000-pg	19.44	19.12	0.32	0.22
	1,000-pg	22.78	21.91	0.88	0.62
	100-pg	26.31	24.20	2.11	1.49
	10-pg	29.73	27.31	2.42	1.71
	1-pg	33.42	29.68	3.74	2.64

Note: C_t values are based on mean triplicates results for *Salmonella* spp. (S) and *Campylobacter* spp. (C) DNA amplification

5.3.4.2 Validation of qPCR assay for the analysis of environmental samples

In environmental and food microbiology, standard curves are used to quantify the concentrations of target genes in diverse samples to mitigate bacterial contamination (Brankatschk et al., 2012). Biological and food samples contain inhibitory substances that are not found in standards but are based on purified templates (Schrader et al., 2012). The inhibitors can reduce PCR efficiency and potentially lead to false negative results (Kubista et al., 2006; Schrader et al., 2012). Some examples of inhibitors include dead biomass and soil that contains humic and fulminic acids; sludge, containing fats, proteins, polyphenols, and heavy metals; waste water containing polysaccharides, metal ions, and RNases; and food containing fats, glycogen, polysaccharides, minerals and enzymes (Schrader et al., 2012).

Constructed standard curves for environmental and food analysis are often based on CFU/mL. For example, Leblanc-Maridor, Beaudeau, Seegers, Denis, and Belloc (2011) validated a qPCR method to quantify *C. coli* and *C. jejuni* in faeces, feed and environmental samples. The standard curve of the assay was constructed by inoculating *Campylobacter* spp. negative samples with 10-fold dilutions of *Campylobacter* spp. suspensions of each reference strains. They proved that the established qPCR assay was highly specific and were over a linear range (from 10^2 to 10^7 CFU, 10^2 to 10^8 CFU and 10^3 to 10^7 CFU for faecal, feed and environmental samples respectively) despite the presence of PCR inhibitors in the sample. Hyeon et al. (2010) also validated and developed a multiplex qPCR method to detect *Cronobacter* spp. and *S. enterica* in powdered infant formula. They inoculated overnight cultures that were serially diluted into powdered infant formula free of *Salmonella* spp. and *Cronobacter* spp. A standard curve was then constructed by plotting C_t versus log of CFU, and a detection limit of 10^3 CFU/g for both species was observed.

In this study, CFU/mL standard curves were generated from inoculated samples to assess PCR inhibitors. Standard curves were generated from ten-fold serial dilutions in 1 % peptone water, covering a range of 1 to 10 Log_{10} CFU/mL per reaction of each pathogen (determined by plate count method) (Elizaquível, Gabaldón, & Aznar, 2011). One-mL of

each dilution stock was inoculated into *Salmonella* spp. and *Campylobacter* spp. negative samples. The inoculated sample suspensions were extracted three times to control for DNA loss, and each extraction were run in triplicate to control for the validity of the qPCR assay. Average C_t values of detectable DNA concentrations for the corresponding Log_{10} CFU/mL were then calculated to construct the standard curve. C_t values greater than 40 or samples with C_t values higher than the negative controls were considered negative in the assay (Gordillo et al., 2014).

The multiplex qPCR assay of inoculated *Salmonella* spp. and *Campylobacter* spp. target in environmental samples were observed to be species-specific. In Figure 5.16, *Salmonella* spp. ($y = -3.2459x + 46.315$, $R^2 = 0.99$) and *Campylobacter* spp. ($y = -3.6953x + 45.815$ with $R^2 = 0.99$) DNA amplification resulted in a linear relationship between C_t and CFU/mL, and an amplification efficiency of 103.27 % and 86.47 %. The detection limit of each assay was determined to be 3.24 - 8.24 Log_{10} CFU/mL for *Salmonella* spp., and 2.97 - 7.97 Log_{10} CFU/mL for *Campylobacter* spp.

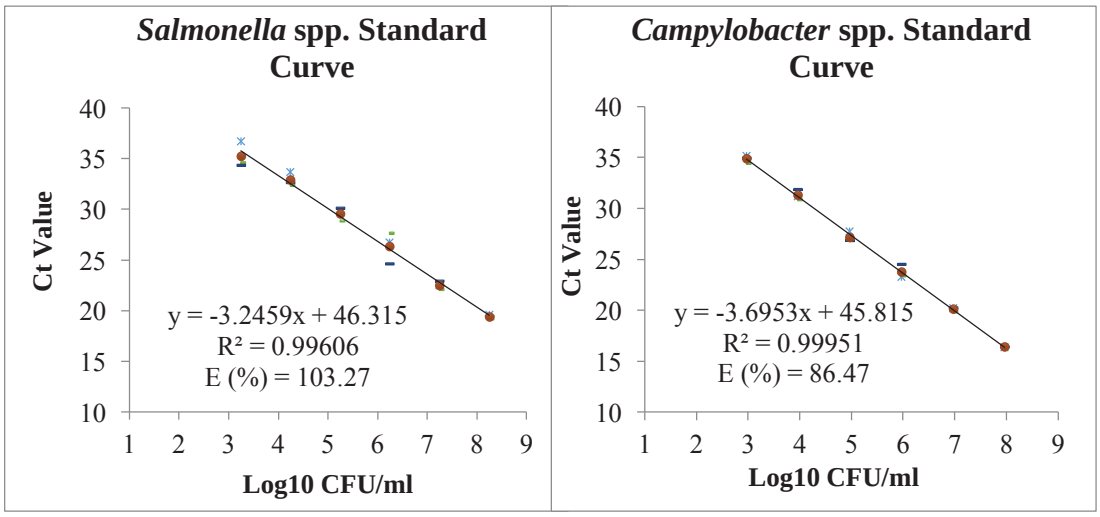

Figure 5.16 Standard curves of inoculated samples *Salmonella* spp. and *Campylobacter* spp.

Note: Serial dilutions of each standard ranged from 2 – 9 Log_{10} CFU/mL per reaction. Efficiency (E) was calculated based on the slope determined by linear regression analysis. Slope and R^2 were calculated based on average results from triplicate data sets of both DNA extraction and qPCR analysis

5.3.5 Agreement between Plating and qPCR method

The agreement between the standard and qPCR method indicates the validity of the quantification data produced by qPCR (Clais et al., 2015). The quantification data of *S. aureus* in environmental samples determined by plate count method (Log_{10} CFU/mL) was comparable to the estimated qPCR data (Log_{10} CFU/mL). The quantification of samples through qPCR was calculated by the construction of standard curves from inoculated samples. A linear equation (y = -3.6701x + 47.456) from the standard curve was then used to calculate the estimated Log_{10} CFU/mL of each sample.

The data gathered from both methods was expected to lie across the identity line and to be normally distributed (Clais et al., 2015). In this study, the samples quantified by the plate count and qPCR method ranged from 1 to 6.1 Log_{10} CFU/mL, and 3.5 to 6 Log_{10} CFU/mL, respectively. Figure 5.17 shows agreement between both methods when the bacterial concentration was above 3.5 Log_{10} CFU/mL. Below this concentration, the agreement between the methods diminishes because the points in the scatter plot do not lie across the identity line (Bland & Altman, 1995). In this study, qPCR equivalents were observed to have higher Log_{10} counts at 3 – 4 Log_{10} CFU/mL, when the plate count method results were 1 – 2 Log_{10} CFU/mL. Similar results were observed from other studies, where qPCR estimates were reported to be higher than standard methods (Botaro et al., 2013; Botteldoorn et al., 2008; De Carvalho, Goncalves, Botaro, Silva, & dos Santos, 2015; Hein, Flekna, Krassnig, & Wagner, 2006)**.** The high concentration on qPCR estimates may be attributed to several factors such as DNA from dead cells, presence of viable but non-cultural forms (which cannot be quantified by plate counts), and one CFU on a plate may be generated by more than one cell (Postollec et al., 2011).

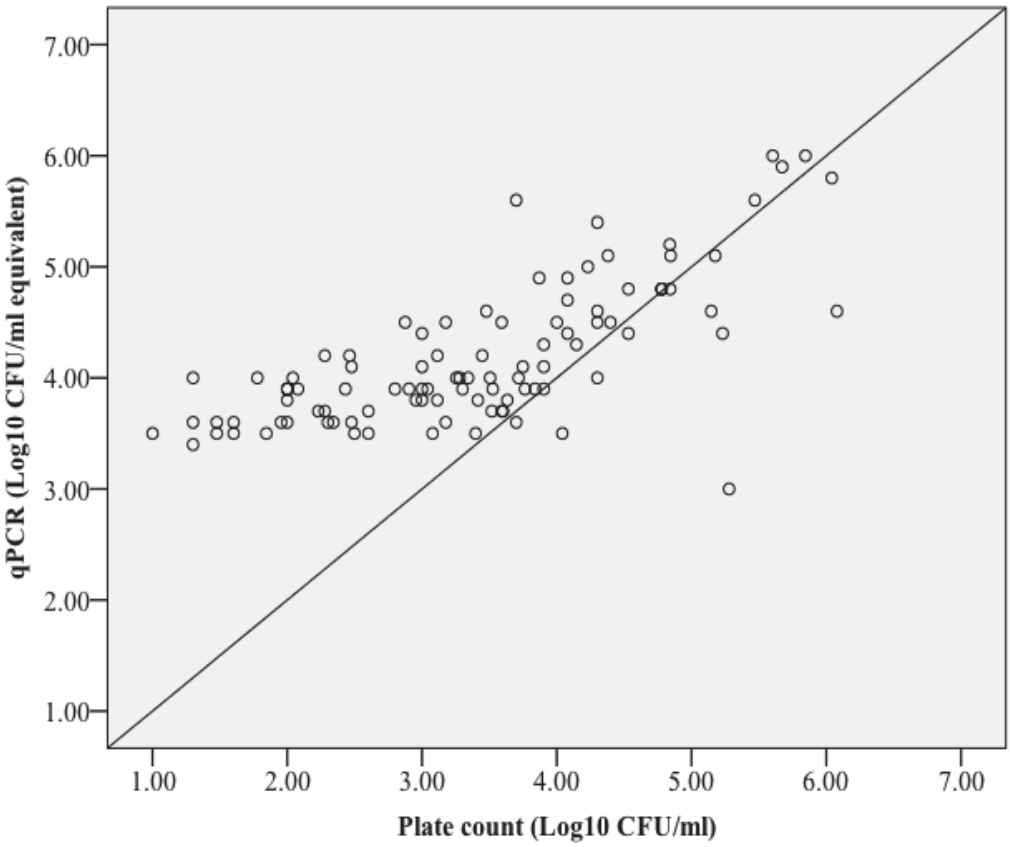

Figure 5.17 Enumeration of *S. aureus* (Log_{10} CFU/mL) obtained by qPCR and the plate count method (Log_{10} CFU/mL) plotted with on an identity line (y=x)

The Bland-Altman plot between plate count and qPCR method was also evaluated to assess differences between the methods. Figure 5.18 shows the mean difference of -0.79 Log_{10} CFU/mL between plate count and qPCR methods, with a 95% confidence interval (limits of agreement) of 0.90 to -2.47 Log_{10} CFU/mL. However, most points in the plot were observed to be within the limits of agreement, whilst the mean discrepancy between the methods were observed to be less than 1 C_t. The results indicated a slight agreement between the two methods. Therefore, qPCR enumeration may be used to estimate the bacterial concentration of samples.

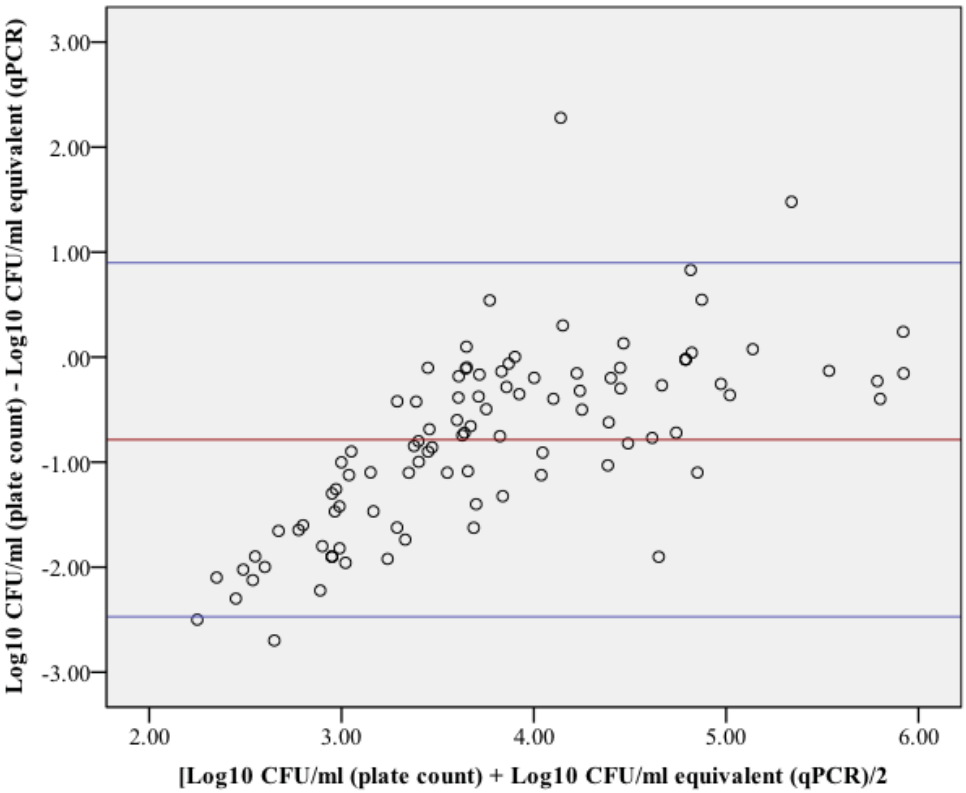

Figure 5.18 Bland-Altman analysis between qPCR and plate count methods

Note: Bland-Altman plot represents the average of the enumeration results (Log_{10} CFU/mL) as a function of the differences observed between qPCR and plate count methods. The bias (red line) is the average (-0.79) difference between the data of both methods. The limits of agreement (blue line) between the two methods ranged from 0.90 to -2.47 (Mean ± (1.96 × SD))

5.3.6 Enumeration of *Salmonella* spp. and *Campylobacter* spp. using multiplex qPCR

Positive samples containing *Salmonella* spp. and *Campylobacter* spp. analysed by standard methods were further enumerated using multiplex qPCR. Linear regression equations: y = -3.2459x + 46.315 (*Salmonella* spp.) and y = -3.6953x + 45.815 (*Campylobacter* spp.) derived from standard curves generated in section 5.3.4 were used to estimate the concentration of the pathogens using equation x:

$$Y = (10^{((X-c)/m)}) \times 10 \quad \text{.....[x]}$$

Y = estimated Log_{10} CFU/mL
X = C_t value of the sample derived from qPCR analysis
c = intercept
m = slope for X

The swab samples were collected on the annex floor (n = 96), crevices (n = 120), drinkers (n = 120), feed loaders (n = 72), feeders (n = 120), fans (n = 96), and vents (n = 120), during three cycles of the cleaning regime (n = 744). Estimated mean counts of *Salmonella* spp. and *Campylobacter* spp. by qPCR, from each location are summarised in Table 5.13. Before initial cleaning, estimated mean counts of *Salmonella* spp. and *Campylobacter* spp., ranged from 3.57 to 3.67 Log_{10} CFU/mL and 3.24 to 3.93 Log_{10} CFU/mL, respectively. After disinfection, *Salmonella* spp. was not detected in samples collected from the fans. Meanwhile, comparing before cleaning and after disinfection, *Salmonella* spp. counts had the lowest Log_{10} decrease in feed loaders (0.08 Log_{10} CFU/mL), annex (0.14 Log_{10} CFU/mL), crevices (0.18 Log_{10} CFU/mL), feeders (0.33 Log_{10} CFU/mL), drinkers (0.5 Log_{10} CFU/mL) and vents (0.5 Log_{10} CFU/mL). Compared to *Salmonella* spp., *Campylobacter* spp. was detected in samples from fans after disinfection, with a decrease of 1.05 Log_{10} CFU/mL. Additionally, feeders had the highest decrease of cell counts (1.12 Log_{10} CFU/mL) after disinfection, followed by the annex (0.57 Log_{10} CFU/mL), drinkers (0.8 Log_{10} CFU/mL), crevices (0.54 Log_{10} CFU/mL), feed loaders (0.75 Log_{10} CFU/mL), and vents (0.35 Log_{10} CFU/mL). The comparison of cell counts before cleaning and after disinfection were not significant on each location.

Therefore, the results from this study suggests that the cleaning regime was not effective in reducing cell counts of *Salmonella* spp. and *Campylobacter* spp.

As discussed in Section 5.3.5, qPCR can accurately determine microbial cell counts if the concentration of the samples is within the detection limits (Rothrock, Cook, & Bolster, 2009; Ruijter et al., 2009). In this study, the microbial counts generated by qPCR after disinfection were below the detection limit of the technique (section 5.3.4). This result may be attributed to poor yield of DNA from the microorganisms, which may be caused by initial low bacterial load (Postollec et al., 2011). Thus, a pre-enrichment step of the test sample has been suggested as one way of increasing DNA in bacteria, thereby improving the efficiency of the method (De Boer, Rahaoui, Leer, Montijn, & van der Vossen, 2015; De Oliveira, Ribeiro, Bergamini, & De Martinis, 2010).

Table 5.13 Mean counts of *Salmonella* spp. and *Campylobacter* spp. analysed by multiplex qPCR

Locations	Stage of cleaning	Mean Log_{10} CFU/mL (positive samples %)	
		Salmonella	*Campylobacter*
Annex	BC	3.61 (18.8)	3.54 (20.8)
	AD	3.47 (2.1)	2.97 (4.2)
Crevices	BC	3.57 (26.7)	3.41 (25)
	AD	3.39 (13.3)	2.87 (15)
Drinkers	BC	3.67 (28.3)	3.62 (38.3)
	AD	3.17 (11.7)	2.82 (13.3)
Feed Loaders	BC	3.57 (22.2)	3.24 (25)
	AD	3.49 (5.6)	2.49 (8.3)
Feeders	BC	3.6 (16.7)	3.46 (20)
	AD	3.27 (5)	2.34 (6.7)
Fans	BC	3.50 (18.8)	3.82 (25)
	AD	ND	2.77 (4.2)
Vents	BC	3.59 (21.7)	3.93 (23.3)
	AD	3.09 (11.7)	3.57 (13.3)

Note: Mean prevalence of positive samples (n = 744) collected from four sheds over three consecutive cycles. Note: ND = not detected; BC = before cleaning; AD = after disinfection

Previous studies have improved the detection of low bacterial concentration in food by including a pre-enrichment step (De Boer et al., 2015; Ibekwe & Grieve, 2003; Malorny et al., 2008). Ibekwe and Grieve (2003) improved the detection and quantification of *E. coli* O157:H7 in environmental samples by combining a 16-hour enrichment with the qPCR analysis, which increased the detection limit from 2.6×10^4 CFU/g^{-1} to < 10 CFU/g^{-1}. Josefsen et al. (2004) reported similar results during the enumeration of low *Campylobacter* spp. concentration in chicken rinse samples by including a 20-hour enrichment step under standard conditions before performing qPCR analysis. The use of an enrichment step, along with the qPCR method, dilutes inhibitory substances and produces conditions that favour the growth of target bacterial pathogens to detectable numbers (Ibekwe & Grieve, 2003; Joyner, Wanless, Sinigalliano, & Lipp, 2014).

6 Conclusions

Salmonella spp., *Campylobacter* spp., *S. aureus* and *E. coli* were present in samples obtained pre-cleaning and post-disinfection. Before cleaning, the four pathogens were present at high prevalence and bacterial concentrations in all seven locations. However, of the seven locations sampled in the broiler sheds, crevices and drinkers were the most contaminated after disinfection, probably due to heavy accumulation of organic matter. As expected, the level of contamination after disinfection was influenced by the load of contamination before cleaning. The source of contamination by the pathogens may be linked to the external environment of the shed, where human activity, pests, and the ventilation system can introduce the four pathogens analysed in this study. To reduce contamination, pathogen control measures and proper application of disinfectants are recommended as effective intervention strategies. Results of the Bland-Altman plot of difference for *S. aureus,* showed agreement between plate cell counts of the bacterium and qPCR method. The qPCR standard curves generated as controls determined the quantification limits to be between 3.24 - 8.24 Log10 CFU/mL for *Salmonella* spp., and 2.97 - 7.97 Log10 CFU/mL for *Campylobacter* spp. respectively. Samples collected before cleaning could be quantified by qPCR analysis due to high bacterial load. However, bacterial concentration post-disinfection was below the detection limit of the qPCR assay. Therefore, bacterial pre-enrichment of test samples may be necessary to improve detection of *Salmonella* spp. and *Campylobacter* spp. by qPCR.

7 Recommendations

Proper use of pathogen control measures and execution of the cleaning regime is important to prevent pathogen re-contamination. In this case, the pathogen control measures in the annex, should be improved by using separate boots upon entry into the shed, as well as changing the virkon of the foot-bath at least once a week (Evans & Sayers, 2000). Additionally, sanitising equipment that enters the shed should also be considered, especially the tyres of the tractor that layers the litter.

After applying the disinfectant, it is recommended to flip feeders to allow drip-drying, as well as placing clean litter, one day before placing chicks. To reduce cross-contamination, feeders should be free of liquid which may contain live bacteria (Allen & Newell, 2005). Additionally, broiler sheds should be kept well-maintained, without crevices and cracks.

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9 Appendix

Appendix A: Reagent preparation

Table A1: Reagents prepared for this study

0.85% Physiological Saline	NaCl (MW: 58)	0.85g
	Milli-Q water	1L
NaOH (10N)	NaOH (MW: 40)	40g
~Made in plastic beaker; Make up to volume (100mL) with Milli-Q water.		
0.5M EDTA, pH 8.0	EDTA (MW: 372.24)	18.612g
~ pH is adjusted to 8 with NaOH (10N), and make up to volume (100mL) with Milli-Q water.		
1M Tris HCl, pH 8.0	TrisBase (MW: 121.14)	60.57g
~ pH is adjusted to 8 with analytical grade HCl (12N), and make up to volume (500mL) with Milli-Q water.		
TE Buffer, pH 8.0	0.5M EDTA, pH 8.0	100µl
	1M Tris HCl, pH 8.0	500µl
	Milli-Q water	49.4mL
Lysis Buffer (4x)	1M Tris Cl	20mL
	0.5M EDTA	2mL
	Triton x - 100	12mL
	Milli-Q water	966mL
	*Lysozyme	0.02g/mL
*Lysozome is only added when need to use buffer		
50x TAE	TrisBase (MW: 121.14)	242g
	Glacial acetic acid (17N)	57.1mL
	0.5M EDTA, pH 8.0	100mL
~ Make up to volume (1000ml) with Milli-Q water.		
1x TAE	50x TAE	20mL
	Milli-Q water	980mL

Appendix B: DNA stocks used for PCR/qPCR analysis

Table B1: Initial DNA concentration and DNA purity of bacterial stocks used.

		DNA Conc. (ng/µl)	260/280
***S.* Typhimurium**		478.00	1.92
		477.9	1.91
		483.00	1.89
		485.90	1.91
		485.00	1.88
	Average	*481.96*	*1.90*
C. jejuni		381.60	1.93
		385.60	1.92
		380.40	1.94
		381.60	1.94
		385.10	1.94
	Average	*382.86*	*1.93*
S. aureus		62.10	1.95
		62.30	1.94
		63.50	1.96
		62.30	2.03
		63.20	2.03
	Average	*62.68*	*1.98*
E. coli		269.60	1.95
		257.50	1.97
		258.50	1.98
		258.50	1.96
		259.70	2.00
	Average	*260.76*	*1.97*

Table B2: Calculation of the initial DNA stocks adjusted to 50 ng/µl stock

***Salmonella* spp.**	C1V1 = C2V2
	= 481.96 ng/µl x 100 µl = 50 ng/µl x ?
	$= \frac{48196 \text{ ng/µl}}{50 \text{ ng/µl}} = 963.92$ µl
	= 963.92 µl - 100 µl
	= 863.92 µl autoclaved Milli-Q water added to adjust to 50 ng/µl stock
***Campylobacter* spp.**	C1V1 = C2V2
	= 382.86 ng/µl x 100 µl = 50 ng/µl x ?
	$= \frac{38286 \text{ ng/µl}}{50 \text{ ng/µl}} = 765.72$ µl
	= 765.72 µl - 100 µl
	= 665.72 µl autoclaved Milli-Q water added to adjust to 50 ng/µl stock
S. aureus	C1V1 = C2V2
	= 62.68 ng/µl x 100 µl = 50 ng/µl x ?
	$= \frac{6268 \text{ ng/µl}}{50 \text{ ng/µl}} = 125.36$ µl
	= 125.36 µl - 100 µl
	= 25.36 µl autoclaved Milli-Q water added to adjust to 50 ng/µl stock
***E. coli* O157**	C1V1 = C2V2
	= 260.76 ng/µl x 100 µl = 50 ng/µl x ?
	$= \frac{26076 \text{ ng/µl}}{50 \text{ ng/µl}} = 521.52$ µl
	= 521.52 µl - 100 µl
	= 421.52 µl autoclaved Milli-Q water added to adjust to 50 ng/µl stock

Table B3: Calculation of 50 ng/µl DNA stock adjusted to 5 ng/µl stock

All target bacterial stock at 50 ng/µl	C1V1 = C2V2
	= 50 ng/µl x 5 µl = 5 ng/µl x ?
	$= \frac{250 \text{ ng/µl}}{5 \text{ ng/µl}} = 50$ µl
	= 50 µl - 5 µl
	= 45 µl autoclaved Milli-Q water added to adjust to 5 ng/µl stock

Table B4: Bacteria concentration of stock used to derive Log_{10} based standard curve

	1.00E+05	1.00E+06	1.00E+07	Count (CFU/mL)	Log_{10}
***S.* Tythimurium**		174.3	10.3	1.74E+09	9.24
C. jejuni	92. 7	11. 7		9.27E+07	7.971
S. aureus		61.7	5.7	6.17E+08	8.79
***E. coli* O157**		111		1.11E+09	9.05

Note: Colony counts presented are based on the mean of triplicate counts

Appendix C: Calculations to reconstitute lyophilised primers

Table C1: Resuspension of lyophilised primers to make up 100 µM stock

Organism (target gene)	nM		H20 added to make 100µM stock
***Salmonella* (ttrR)**			
Forward	48	48nM x 1µM/1000nM = 0.048µM 0.048µM/100µM/L = 0.00048L 0.00048L x 1000m/L = 0.48mL or 480µL	480µL
Reverse	34.7	34.7nM x 1µM/1000nM = 0.0347µM 0.0347µM/100µM/L = 0.000347L 0.000347L x 1000m/L = 0.347mL or 347µL	347µL
***Campylobacter* (16s rRNA)**			
Forward	43.4	43.4nM x 1µM/1000nM = 0.0434µM 0.0434µM/100uM/L = 0.000434L 0.000434L x 1000m/L = 0.434mL or 434µL	434µL
Reverse	45.6	45.6nM x 1µM/1000nM = 0.0456µM 0.0456µM/100µM/L = 0.000456L 0.000456L x 1000m/L = 0.456mL or 456µL	456µL
***S. aureus* (htrA)**			
Forward	39.2	39.2nM x 1µM/1000nM = 0.0392µM 0.0392µM/100µM/L = 0.000392L 0.000392L x 1000m/L = 0.392mL or 392µL	392µL
Reverse	42.9	42.9nM x 1µM/1000nM = 0.0429µM 0.0429µM/100µM/L = 0.000429L 0.000429L x 1000m/L = 0.429mL or 429µL	429µL
***E. coli* O157:H7 (eae)**			
Forward	56.2	56.2nM x 1µM/1000nM = 0.0562µM 0.0562µM/100µM/L = 0.000562L 0.000562L x 1000m/L = 0.562mL or 562µL	562µL
Reverse	42.2	42.2nM x 1µM/1000nM = 0.0422µM 0.0422µM/100µM/L = 0.000422L 0.000422L x 1000m/L = 0.422mL or 422µL	422µL

Appendix D: Primer and probe calculations to make 20 µM stocks

Table D1: Calculations for adjusting primer concentrations to 20 µM from 100 µM

	Forward	Reverse
Salmonella **(ttrR)**	C1V1 = C2V2 = 100µM x 480µL = 20µM x ? $= \frac{48000\mu M/\mu l}{20\mu M} = 2400\mu l$ = 2400µl - 480µL = 1920µl TE added to 100µM stock to adjust to 20µM stock	C1V1 = C2V2 = 100µM x 347µL = 20µM x ? $= \frac{34700\mu M/\mu l}{20\mu M} = 1735\mu l$ = 1735µl - 347µL = 1388µl TE added to 100µM stock to adjust to 20µM stock
Campylobacter **(16s rRNA)**	C1V1 = C2V2 = 100µM x 434µL = 20µM x ? $= \frac{43400\mu M/\mu l}{20\mu M} = 2170\mu l$ = 2170µl - 434µL = 1736µl TE added to 100µM stock to adjust to 20µM stock	C1V1 = C2V2 = 100µM x 456µL = 20µM x ? $= \frac{45600\mu M/\mu l}{20\mu M} = 2280\mu l$ 2280µl - 456µL = 1824µl TE added to 100µM stock to adjust to 20µM stock
S. aureus **(htrA)**	C1V1 = C2V2 = 100µM x 392µL = 20µM x ? $= \frac{39200\mu M/\mu l}{20\mu M} = 1960\mu l$ = 1960µl - 392µL = 1568µl TE added to 100µM stock to adjust to 20µM stock	C1V1 = C2V2 = 100µM x 429µL = 20µM x ? $= \frac{42900\mu M/\mu l}{20\mu M} = 2145\mu l$ = 2145µl - 429µL = 1716µl TE added to 100µM stock to adjust to 20µM stock
E. coli **O157:H7 (eae)**	C1V1 = C2V2 = 100µM x 562µL = 20µM x ? $= \frac{56200\mu M/\mu l}{20\mu M} = 2810\mu l$ = 2810µl - 562µL = 2248µl TE added to 100µM stock to adjust to 20µM stock	C1V1 = C2V2 100µM x 422µL = 20µM x ? $= \frac{42200\mu M/\mu l}{20\mu M} = 2110\mu l$ = 2110µl - 422µL = 1688µl TE added to 100µM stock to adjust to 20µM stock

Table D2: Calculations for adjusting probe concentrations to 20 µM stock

All target probes	C1V1 = C2V2 = 100 x 60µL = 20µM x ? $= \frac{6000\mu M/\mu l}{20\mu M} = 300\mu l$ = 300µl - 60µL = 240µl TE added to 100µM stock to adjust to 20µM stock

Appendix E: Primer and probe calculations for adjusting to working stocks.

Table E1: Calculations to make primer (10 µM) and probe (5 µM) working stocks

Primers	Probes
C1V1 = C2V2	C1V1 = C2V2
= 20µM x 50µL = 10µM x ?	= 20µM x 50µL = 5µM x ?
$= \frac{1000\mu M/\mu l}{10\mu M} = 100\mu l$	$= \frac{1000\mu M/\mu l}{5\mu M} = 200$
= 100µl - 50µL	200µl - 50µL
= 50µl TE added to 20µM stock to adjust to 10µM working stock	= 150µl TE added to 20µM stock to adjust to 5µM working stock

Appendix F: Primer and probe concentration calculations used in the reaction mix for PCR/qPCR analysis

Table F1: Calculation of primer concentrations used in the reaction mix

0.9µM c1v1=c2v2
= 10µM x ? = 0.9µM x 20µl
= $\frac{18\mu M/\mu l}{10\mu m}$ =1.8µl

0.4µM c1v1=c2v2
= 10µM x ? = 0.40µM x 20µl
= $\frac{8\mu M/\mu l}{10\mu m}$ =0.8µl

0.8µM c1v1=c2v2
= 10µM x ? = 0.8µM x 20µl
= $\frac{16\mu M/\mu l}{10\mu m}$ =1.6µl

0.35µM c1v1=c2v2
= 10µM x ? = 0.35µM x 20µl
= $\frac{7\mu M/\mu l}{10\mu m}$ =0.7µl

0.7µM c1v1=c2v2
= 10µM x ? = 0.7µM x 20µl
= $\frac{14\mu M/\mu l}{10\mu m}$ =1.4µl

0.3µM c1v1=c2v2
= 10µM x ? = 0.3µM x 20µl
= $\frac{6\mu M/\mu l}{10\mu m}$ =0.6µl

0.6µM c1v1=c2v2
= 10µM x ? = 0.6µM x 20µl
= $\frac{12\mu M/\mu l}{10\mu m}$ =1.2µl

0.25µM c1v1=c2v2
= 10µM x ? = 0.25µM x 20µl
= $\frac{5\mu M/\mu l}{10\mu m}$ =0.5µl

0.5µM c1v1=c2v2
= 10µM x ? = 0.5µM x 20µl
= $\frac{10\mu M/\mu l}{10\mu m}$ =1µl

0.2µM c1v1=c2v2
10µM x ? = 0.20µM x 20µl
= $\frac{4\mu M/\mu l}{10\mu m}$ =0.4µl

0.45µM c1v1=c2v2
= 10µM x ?= 0.45µM x20µl
= $\frac{9\mu M/\mu l}{10\mu m}$ =0.9µl

0.15µM c1v1=c2v2
= 10µM x ? = 0.15µM x 20µl
= $\frac{3\mu M/\mu l}{10\mu m}$ =0.3µl

Table F2: Calculation of probe concentration used in the reactions mix

0.25µM c1v1=c2v2
= 5µM x ? = 0.25µM x 20µl
= $\frac{5\mu M/\mu l}{5\mu m}$ =1µl

Appendix G: DNA concentration calculations used in the reaction mix for PCR/qPCR analysis

Table G1: Calculation of DNA concentration used in the reaction mix

10 ng/ 10000 pg	c1v1=c2v2
=	5 ng/µl x ? = (10 ng/ 20 µl) x 20 µl
=	$\frac{(0.5\ \text{ng/µl} \times 20\ \text{µl})}{5\text{ng/µl}}$ =2 µl
100 ng/ 100000 pg	c1v1=c2v2
=	50 ng/µl x ? = (100 ng/ 20 µl) x 20 µl
=	$\frac{(5\ \text{ng/µl} \times 20\ \text{µl})}{50\ \text{ng/µl}}$ =2 µl

Appendix H: Reaction mix for PCR analysis

Table H1: PCR optimisation reaction mix at 20 µl reaction volume

	Concentration	**Volume**
Master mix (2x)	1x	10µl
Primer (10µM)	900nm	1.8µl
Primer (10µM)	900nm	1.8µl
Milli-Q water		4.4µl
DNA stock (5ng/µl)	10ng	2µl
Master mix (2x)	1x	10µl
Primer (10µM)	500nm	1µl
Primer (10µM)	500nm	1µl
Milli-Q water		6µl
DNA stock (5ng/µl)	10ng	2µl
Master mix (2x)	1x	10µl
Primer (10µM)	300nm	0.6µl
Primer (10µM)	300nm	0.6µl
Milli-Q water		6.8µl
DNA stock (5ng/µl)	10ng	2µl

Appendix I: Reaction mix used for qPCR analysis

Table I1: qPCR reaction mix at 20 µl singleplex and multiplex reaction

	Singleplex		
		Concentration	**Volume**
Target 1/2	Master mix (2x)	1x	10µl
	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl
	Milli-Q water		5µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl
	Multiplex		
		Concentration	**Volume**
	Master mix (2x)	1x	10µl
Target 1	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl
Target 2	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl

Appendix J: Reaction mix used for singleplex qPCR analysis during optimisation

Table J1: qPCR reaction mix at 20 µl singleplex reaction during optimisation

		Concentration	Volume			Concentration	Volume
0.8µM	Master mix (2x)	1x	10µl	**0.35µM**	Master mix (2x)	1x	10µl
	Primer (10µM)	800nm	1.6µl		Primer (10µM)	350nm	0.7µl
	Primer (10µM)	800nm	1.6µl		Primer (10µM)	350nm	0.7µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		3.8µl		Milli-Q water		5.6µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl		DNA stock (50ng/µl)	10000pg - 1pg	2µl
0.7µM	Master mix (2x)	1x	10µl	**0.3µM**	Master mix (2x)	1x	10µl
	Primer (10µM)	700nm	1.4µl		Primer (10µM)	300nm	0.6µl
	Primer (10µM)	700nm	1.4µl		Primer (10µM)	300nm	0.6µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		4.2µl		Milli-Q water		5.8µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl		DNA stock (50ng/µl)	10000pg - 1pg	2µl
0.6µM	Master mix (2x)	1x	10µl	**0.25µM**	Master mix (2x)	1x	10µl
	Primer (10µM)	600nm	1.2µl		Primer (10µM)	250nm	0.5µl
	Primer (10µM)	600nm	1.2µl		Primer (10µM)	250nm	0.5µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		4.6µl		Milli-Q water		6µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl		DNA stock (50ng/µl)	10000pg - 1pg	2µl
0.5µM	Master mix (2x)	1x	10µl	**0.2µM**	Master mix (2x)	1x	10µl
	Primer (10µM)	500nm	1µl		Primer (10µM)	200nm	0.4µl
	Primer (10µM)	500nm	1µl		Primer (10µM)	200nm	0.4µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		5µl		Milli-Q water		6.2µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl		DNA stock (50ng/µl)	10000pg - 1pg	2µl

Note: continues onto the next page

0.45µM	Master mix (2x)	1x	10µl	**0.15µM**	Master mix (2x)	1x	10µl
	Primer (10µM)	450nm	0.9µl		Primer (10µM)	150nm	0.3µl
	Primer (10µM)	450nm	0.9µl		Primer (10µM)	150nm	0.3µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		5.2µl		Milli-Q water		6.4µl
	DNA stock (50ng/µl)	10000pg - 1pg	2µl		DNA stock (50ng/µl)	10000pg - 1pg	2µl
0.4µM	Master mix (2x)	1x	10µl				
	Primer (10µM)	400nm	0.8µl				
	Primer (10µM)	400nm	0.8µl				
	Probe (5µM)	250nm	1µl				
	Milli-Q water		5.4µl				
	DNA stock (50ng/µl)	10000pg - 1pg	2µl				

Appendix K: Reaction mix used for multiplex qPCR analysis during optimisation

Table K1: qPCR reaction mix at 20 µl multiplex reaction during optimisation 1 of *E. coli* O157:H7 (E) and *Campylobacter* spp. (C)

		Concentration	**Volume**			**Concentration**	**Volume**
	Master mix (2x)	1x	10µl		Master mix (2x)	1x	10µl
E at 0.5µM	Primer (10µM)	500nm	1µl	**E at 0.5µM**	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl		Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
C at 0.45µM	Primer (10µM)	450nm	0.9µl	**C at 0.40µM**	Primer (10µM)	400nm	0.8µl
	Primer (10µM)	450nm	0.9µl		Primer (10µM)	400nm	0.8µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		0.2µl		Milli-Q water		0.4µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl		DNA stock (50ng/µl)	1000pg - 1pg	4µl

Table K2: qPCR reaction mix at 20 µl multiplex reaction during optimisation 2 of *E. coli* O157:H7 (E) and *Campylobacter* spp. (C)

		Concentration	Volume			Concentration	Volume
	Master mix (2x)	1x	10µl		Master mix (2x)	1x	10µl
E at 0.5µM	Primer (10µM)	500nm	1µl	**E at 0.5µM**	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl		Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
C at 0.35µM	Primer (10µM)	350nm	0.7µl	**C at 0.30µM**	Primer (10µM)	300nm	0.6µl
	Primer (10µM)	350nm	0.7µl		Primer (10µM)	300nm	0.6µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		0.6µl		Milli-Q water		0.8µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl		DNA stock (50ng/µl)	1000pg - 1pg	4µl
	Master mix (2x)	1x	10µl		Master mix (2x)	1x	10µl
E at 0.5µM	Primer (10µM)	500nm	1µl	**E at 0.5µM**	Primer (10µM)	500nm	1µl
	Primer (10µM)	500nm	1µl		Primer (10µM)	500nm	1µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
C at 0.25µM	Primer (10µM)	250nm	0.5µl	**C at 0.20µM**	Primer (10µM)	200nm	0.4µl
	Primer (10µM)	250nm	0.5µl		Primer (10µM)	200nm	0.4µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		1µl		Milli-Q water		1.2µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl		DNA stock (50ng/µl)	1000pg - 1pg	4µl
	Master mix (2x)	1x	10µl				
E at 0.5µM	Primer (10µM)	500nm	1µl				
	Primer (10µM)	500nm	1µl				
	Probe (5µM)	250nm	1µl				
C at 0.15µM	Primer (10µM)	150nm	0.3µl				
	Primer (10µM)	150nm	0.3µl				
	Probe (5µM)	250nm	1µl				
	Milli-Q water		1.4µl				
	DNA stock (50ng/µl)	1000pg - 1pg	4µl				

Table K3: qPCR reaction mix at 20 µl multiplex reaction during optimisation 3 of *E. coli* O157:H7 (E) and *Campylobacter* spp. (C)

		Concentration	Volume			Concentration	Volume
	Master mix (2x)	1x	10µl		Master mix (2x)	1x	10µl
C at 0.15µM	Primer (10µM)	150nm	0.3µl	**C at 0.15µM**	Primer (10µM)	150nm	0.3µl
	Primer (10µM)	150nm	0.3µl		Primer (10µM)	150nm	0.3µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
E at 0.30µM	Primer (10µM)	300nm	0.6µl	**E at 0.40µM**	Primer (10µM)	400nm	0.8µl
	Primer (10µM)	300nm	0.6µl		Primer (10µM)	400nm	0.8µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		2.2µl		Milli-Q water		1.8µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl		DNA stock (50ng/µl)	1000pg - 1pg	4µl
	Master mix (2x)	1x	10µl		Master mix (2x)	1x	10µl
C at 0.15µM	Primer (10µM)	150nm	0.3µl	**C at 0.15µM**	Primer (10µM)	150nm	0.3µl
	Primer (10µM)	150nm	0.3µl		Primer (10µM)	150nm	0.3µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
E at 0.60µM	Primer (10µM)	600nm	1.2µl	**E at 0.70µM**	Primer (10µM)	700nm	1.4µl
	Primer (10µM)	600nm	1.2µl		Primer (10µM)	700nm	1.4µl
	Probe (5µM)	250nm	1µl		Probe (5µM)	250nm	1µl
	Milli-Q water		1µl		Milli-Q water		0.6µl
	DNA stock (50ng/µl)	1000pg - 1pg	4µl		DNA stock (50ng/µl)	1000pg - 1pg	4µl
	Master mix (2x)	1x	10µl				
C at 0.15µM	Primer (10µM)	150nm	0.3µl				
	Primer (10µM)	150nm	0.3µl				
	Probe (5µM)	250nm	1µl				
C at 0.80µM	Primer (10µM)	800nm	1.6µl				
	Primer (10µM)	800nm	1.6µl				
	Probe (5µM)	250nm	1µl				
	Milli-Q water		0.2µl				
	DNA stock (50ng/µl)	1000pg - 1pg	4µl				

Appendix L: Singleplex qPCR DNA amplification plot of *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* O157:H7

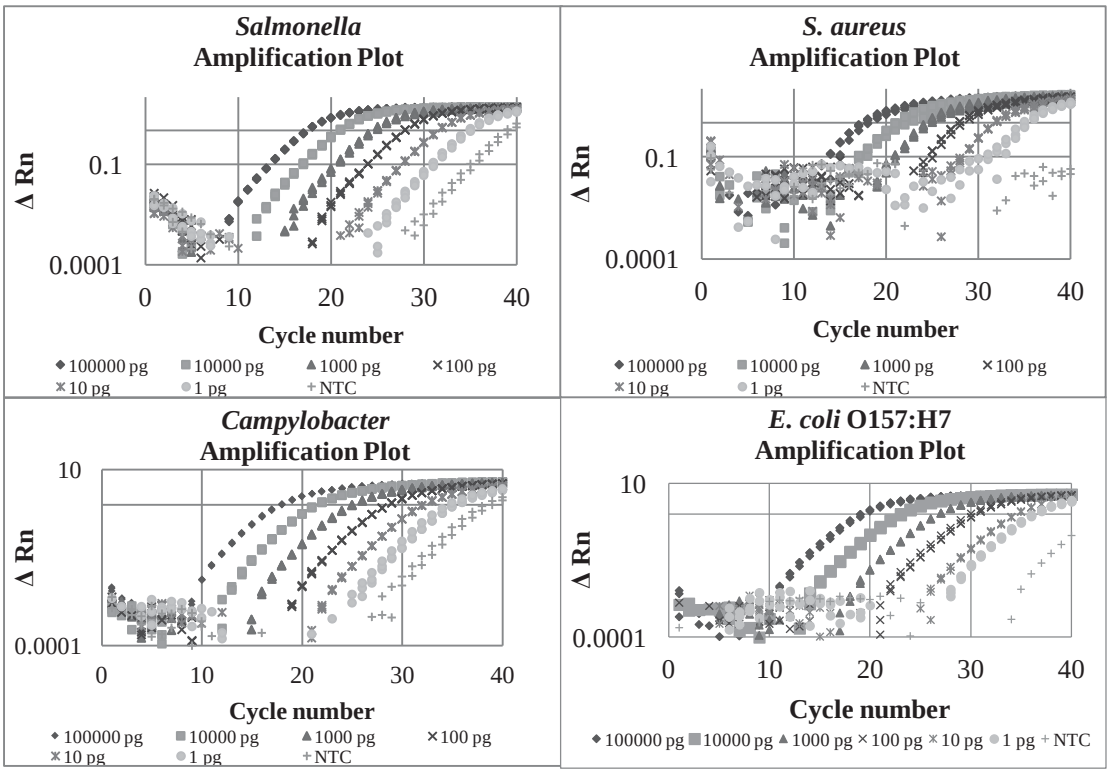

Figure L1: *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* O157:H7 singleplex qPCR amplification plot (10000pg-1pg). NOTE: NTC = No template control (negative control – Milli-Q water); n = triplicates

Appendix M: Singleplex and multiplex qPCR DNA amplification plot of *Campylobacter* spp., *Salmonella* spp., *S. aureus* and *E. coli* O157:H7

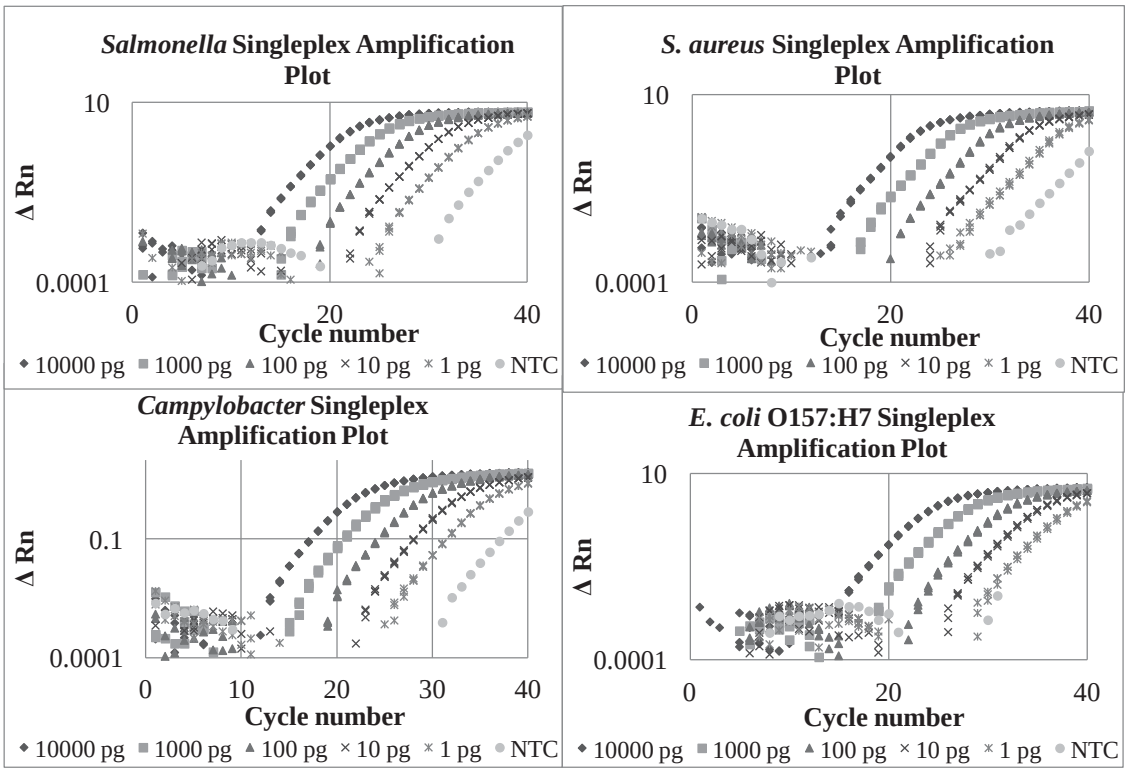

Figure M1: *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* O157:H7 singleplex qPCR amplification plot (10000pg-1pg). NOTE: NTC = No template control (negative control – Milli-Q water); n = triplicates

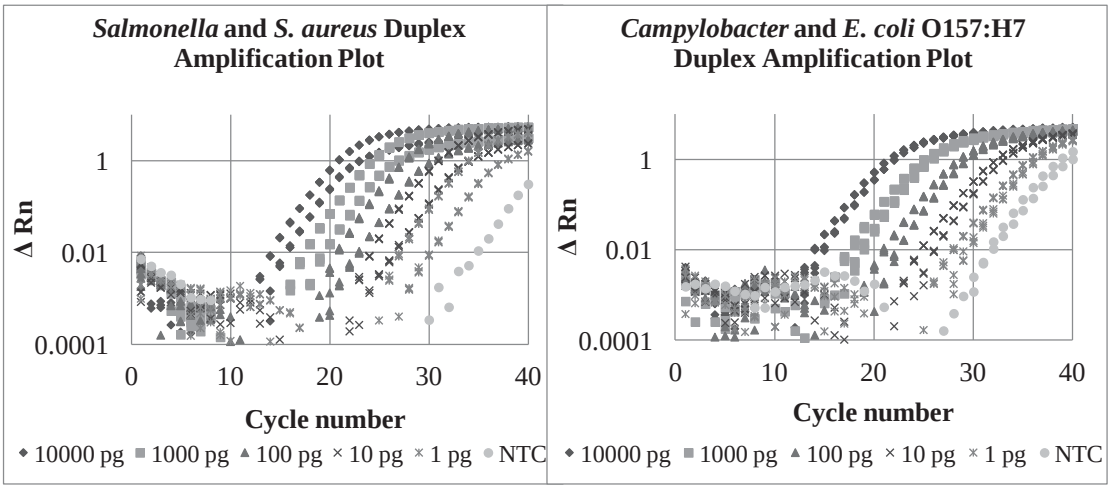

Figure M2: *Salmonella* spp., *Campylobacter* spp., *S. aureus* and *E. coli* O157:H7 singleplex qPCR amplification plot (10000pg-1pg). NOTE: NTC = No template control (negative control – Milli-Q water); n = triplicates

Appendix N: Singleplex and multiplex qPCR reaction of *Campylobacter* spp. and *Salmonella* spp.

Table N1: Singleplex and Multiplex qPCR

		Singleplex Ct (threshold cycle) *DNA Concentration (pg)*					**Multiplex** Ct (threshold cycle) *DNA Concentration (pg)*				
		10000	**1000**	**100**	**10**	**1**	**10000**	**1000**	**100**	**10**	**1**
Campylobacter	**1**	19.40	23.90	27.17	30.35	32.50	19.58	23.28	26.92	30.51	33.64
	2	19.26	23.87	27.20	29.90	33.10	19.10	23.58	26.84	30.54	32.67
	3	19.55	23.45	27.21	29.89	33.17	19.25	23.30	26.85	30.48	32.70
	Average	19.40	23.74	27.19	30.05	32.92	19.31	23.39	26.87	30.51	33.00
	SD	0.15	0.25	0.02	0.26	0.37	0.25	0.17	0.04	0.03	0.55
Salmonella	**1**	18.59	21.82	25.24	28.59	31.60	18.38	22.28	25.45	28.67	30.70
	2	18.54	21.93	25.24	28.69	31.83	18.83	22.19	25.40	28.82	31.54
	3	18.55	21.90	25.20	28.50	31.80	18.81	22.20	25.50	28.70	31.64
	Average	18.56	21.88	25.23	28.59	31.74	18.67	22.22	25.45	28.73	31.29
	SD	0.03	0.06	0.02	0.10	0.13	0.25	0.05	0.05	0.08	0.52

Note: Average ± SD is based on triplicates of *Campylobacter* spp. and *Salmonella* spp. DNA amplification

Appendix O: Singleplex and multiplex qPCR reaction of *Salmonella* spp. and *Campylobacter* spp. in inoculated samples

Table O1: Multiplex qPCR of *Salmonella* spp. target with *Campylobacter* spp.

	Salmonella					
	Log$_{10}$ CFU/mL					
	8.24	**7.24**	**6.24**	**5.24**	**4.24**	**3.24**
	19.72	22.55	26.52	29.46	33.55	36.88
	19.64	22.52	27.04	29.85	33.82	37.01
	19.42	22.4	26.62	29.96	33.69	36.23
Average	19.59	22.49	26.73	29.76	33.69	36.71
SD	0.155	0.079	0.276	0.263	0.135	0.418
	18.92	22.02	25.5	28.91	32.55	34.23
	19.97	22.17	25.68	28.9	32.24	34.45
	18.79	22.12	25.32	28.84	32.55	35.29
Average	19.23	22.10	27.71	28.88	32.45	34.66
SD	0.647	0.076	0.18	0.038	0.179	0.559
	19.35	22.81	24.1	30.28	32.79	34.45
	19.61	23.26	24.9	30.15	32.69	34.23
	19.42	22.8	24.99	30.05	32.46	34.5
Average	19.46	22.96	24.66	30.16	32.65	34.39
SD	0.135	0.263	0.49	0.115	0.169	0.144

Note: Average ± SD is based on triplicates of *Salmonella* spp. DNA amplification.

Table O2: Multiplex qPCR of *Campylobacter* spp. *target* with *Salmonella* spp.

	Campylobacter					
	Log_{10} CFU/mL					
	7.97	**6.97**	**5.97**	**4.97**	**3.97**	**2.97**
	16.51	20.14	23.49	27.73	31.43	35.32
	16.31	20.2	23.41	27.81	30.94	35.15
	16.28	20.26	23.01	27.72	31.24	34.95
Average	16.37	20.20	23.30	27.75	31.20	35.14
SD	0.125	0.06	0.257	0.049	0.247	0.185
	16.48	19.97	23.29	26.97	30.81	34.36
	16.44	19.86	23.54	26.9	31.01	34.55
	16.43	19.89	23.45	26.61	30.84	34.55
Average	16.45	19.91	23.43	26.83	30.89	34.49
SD	0.026	0.057	0.127	0.191	0.108	0.11
	16.4	20.42	24.55	26.97	32.09	34.94
	16.42	20.13	24.63	26.95	32.14	34.9
	16.5	20.17	24.44	26.92	31.49	35.14
Average	16.44	20.24	24.54	26.95	31.91	34.99
SD	0.053	0.157	0.095	0.025	0.362	0.129

Note: Average ± SD is based on triplicates of *Campylobacter* DNA amplification.

Appendix P: Singleplex qPCR reaction of *S. aureus* in inoculated samples

Table P1: Singleplex qPCR of *S. aureus* target

	S. aureus						
	Log_{10} CFU/mL						
	8.8	**7.8**	**6.8**	**5.8**	**4.8**	**3.8**	**2.8**
	14.16	17.65	23.6	28.01	31.49	33.48	35.72
	14.26	18.17	23.44	27.91	31.36	33.4	35.98
	14.45	18	23.32	27.89	31.38	33.4	36.26
Average	14.29	14.29	17.94	27.94	31.41	33.43	35.99
SD	0.147	0.265	0.14	0.064	0.07	0.046	0.27
	14.29	17.83	23.7	27.16	31.34	32.66	35.95
	14.56	17.72	23.54	27.32	30.74	32.46	36.3
	14.37	17.72	23.54	26.96	30.99	32.66	36.87
Average	14.41	17.76	23.59	27.15	31.02	32.59	36.37
SD	0.139	0.064	0.092	0.18	0.301	0.115	0.46
	14.33	17.66	23.63	27.48	30.8	32.75	35.58
	14.5	17.91	23.59	27.55	30.73	32.68	35.86
	14.57	18.02	23.62	27.56	30.79	33.31	35.98
Average	14.47	17.86	23.61	27.53	30.77	32.91	35.81
SD	0.123	0.184	0.021	0.044	0.038	0.345	0.21

Note: Average ± SD is based on triplicates of *S. aureus* DNA amplification.

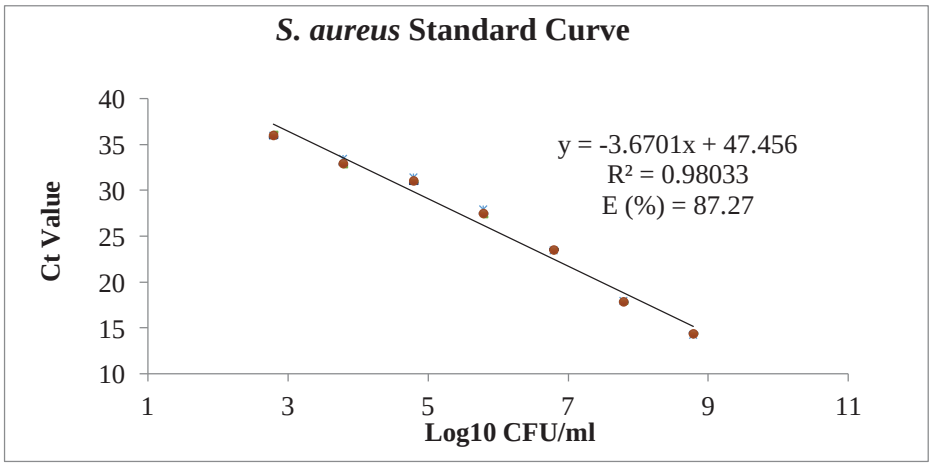

Figure P1: *S. aureus* standard curves from inoculated samples. NOTE: Serial dilutions of each standard ranged from 2 – 9 Log_{10} CFU/mL per reaction. Efficiency (E) was calculated based on the slope determined by linear regression analysis. Slope and R^2 were calculated based on the average results from triplicate data sets of both DNA extraction and qPCR analysis.

Appendix Q: Quantified qPCR estimate of *S. aureus*

Table Q1: Quantification of qPCR estimates for *S. aureus* target

			Log_{10} CFU/mL	
Sample ID	Ct	(10^((Ct-c)/m)) *10	qPCR	Plate Count
276	29.4	814570.8	5.9	5.7
277	34.4	36171.7	4.6	4.3
284	35.1	22989.6	4.4	3.0
285	34.9	12809.4	4.1	3.0
286	36.9	7337.5	3.9	3.9
287	36.4	10071.6	4.0	3.3
288	38.2	3356.7	3.5	2.6
296	29.1	998180.2	6.0	5.6
374	35.7	15991.3	4.2	3.1
375	34.9	25570.0	4.4	4.5
376	34.7	30065.1	4.5	3.6
378	36.3	10790.4	4.0	3.7
387	35.3	20195.4	4.3	3.9
388	38.3	3131.2	3.5	3.4
390	37.9	4076.2	3.6	3.2
391	36.9	7364.8	3.9	2.9
392	36.8	8009.1	3.9	3.8
393	38.2	3323.4	3.5	3.1
394	35.4	18730.3	4.3	4.1
501	36.5	9514.8	4.0	4.3
502	34.6	31039.4	4.5	4.3
505	34.6	31336.7	4.5	4.4
506	34.0	46552.2	4.7	4.1
508	38.0	3667.4	3.6	2.3
507	38.2	3393.9	3.5	4.0
510	33.1	79334.3	4.9	4.1
513	39.9	1112.9	3.0	5.3
514	32.5	116470.5	5.1	4.4
515	32.0	162412.1	5.2	4.8
517	32.9	92857.6	5.0	4.2
518	33.2	74436.0	4.9	3.9
519	29.9	602202.1	5.8	6.0

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Sample ID	Ct	(10^((Ct-c)/m)) *10	Log_{10} CFU/mL	
			qPCR	Plate Count
520	32.4	123103.9	5.1	4.8
521	34.3	39094.8	4.6	5.1
522	34.2	41526.9	4.6	6.1
523	34.9	26092.1	4.4	5.2
524	33.6	58409.9	4.8	4.8
525	30.7	356846.0	5.6	5.5
526	33.4	69585.0	4.8	4.8
528	37.8	4273.8	3.6	3.7
531	37.0	7257.4	3.9	3.3
625	37.8	4315.8	3.6	2.0
627	37.7	4438.2	3.6	1.6
628	36.9	7527.7	3.9	2.4
629	36.8	8198.5	3.9	2.1
632	37.6	4932.4	3.7	3.6
633	37.5	5239.5	3.7	3.6
635	36.1	12158.3	4.1	2.5
636	34.5	34503.6	4.5	4.0
637	36.6	8850.8	3.9	2.0
641	38.3	3168.5	3.5	1.8
642	38.0	3768.9	3.6	2.0
643	36.5	9460.1	4.0	1.8
644	37.1	6640.1	3.8	3.0
649	31.3	244746.7	5.4	4.3
650	36.8	8168.6	3.9	2.0
651	30.6	398242.1	5.6	3.7
653	38.2	3230.1	3.5	1.0
654	38.5	2766.7	3.4	1.3
687	38.3	3030.1	3.5	1.6
688	38.4	2865.9	3.5	1.5
689	36.6	9180.3	4.0	2.0
691	36.4	10185.2	4.0	1.3
692	36.2	11903.2	4.1	3.7
693	36.7	8795.7	3.9	2.8
696	36.8	7950.3	3.9	3.0
697	37.8	4264.6	3.6	2.3

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Sample ID	Ct	(10^((Ct-c)/m)) *10	Log_{10} CFU/mL	
			qPCR	Plate Count
698	38.0	3671.2	3.6	2.5
700	33.5	63544.7	4.8	4.8
701	36.4	10180.0	4.0	3.3
702	36.7	8535.0	3.9	3.5
703	35.9	14256.9	4.2	2.3
704	38.2	3330.8	3.5	2.5
705	37.4	5571.0	3.7	2.2
707	37.4	5471.6	3.7	2.3
708	37.2	6169.0	3.8	3.1
709	36.8	8228.5	3.9	3.0
710	35.0	24085.6	4.4	4.1
711	37.1	6778.3	3.8	3.0
721	36.6	8878.2	3.9	2.0
722	34.6	31202.7	4.5	2.9
728	38.0	3806.2	3.6	1.3
731	37.7	4518.6	3.7	3.5
732	34.4	35062.7	4.5	3.2
733	34.4	36602.6	4.6	3.5
734	29.2	948572.1	6.0	5.8
735	35.6	16867.5	4.2	3.4
736	37.2	6068.6	3.8	3.4
737	37.5	5267.8	3.7	2.6
738	38.0	3752.6	3.6	1.5
739	35.8	15394.4	4.2	2.5
740	36.5	9958.6	4.0	3.5
741	37.0	7254.1	3.9	3.8
742	37.0	6935.4	3.8	2.0
743	37.2	6105.8	3.8	3.6
744	36.6	9154.7	4.0	3.3
745	33.5	61975.1	4.8	4.8
746	33.5	64971.1	4.8	4.5
747	32.4	129227.8	5.1	5.2
748	36.2	11908.0	4.1	3.9

Note: *S. aureus* enumeration for qPCR Log_{10} CFU/mL equivalent was derived from the linear equation y = -3.6701x + 47.456 of inoculated samples (control).

Appendix R: One sample T-test for Bland-Altman plot of difference

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Diff	100	-.7860	.85995	.08599

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Diff	-9.140	99	.000	-.78600	-.9566	-.6154

Appendix S: Cross-tabulation of *Salmonella* spp. and *Campylobacter* spp. prevalence on each location of the shed

***Salmonella* Annexe Cross-tabulation**

Cycle No				After		
				Absent	Present	Total
Cycle1	Before	Absent	Count	11		11
			% of Total	68.8%		68.8%
		Present	Count	5		5
			% of Total	31.3%		31.3%
	Total		Count	16		16
			% of Total	100.0%		100.0%
Cycle2	Before	Absent	Count	13		13
			% of Total	81.3%		81.3%
		Present	Count	3		3
			% of Total	18.8%		18.8%
	Total		Count	16		16
			% of Total	100.0%		100.0%
Cycle3	Before	Absent	Count	15	0	15
			% of Total	93.8%	0.0%	93.8%
		Present	Count	0	1	1
			% of Total	0.0%	6.3%	6.3%
	Total		Count	15	1	16
			% of Total	93.8%	6.3%	100.0%
Total	Before	Absent	Count	39	0	39
			% of Total	81.3%	0.0%	81.3%
		Present	Count	8	1	9
			% of Total	16.7%	2.1%	18.8%
	Total		Count	47	1	48
			% of Total	97.9%	2.1%	100.0%

Salmonella Crevices Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	8	0	8
			% of Total	40.0%	0.0%	40.0%
		Present	Count	6	6	12
			% of Total	30.0%	30.0%	60.0%
	Total		Count	14	6	20
			% of Total	70.0%	30.0%	100.0%
Cycle2	Before	Absent	Count	19		19
			% of Total	95.0%		95.0%
		Present	Count	1		1
			% of Total	5.0%		5.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Cycle3	Before	Absent	Count	17	0	17
			% of Total	85.0%	0.0%	85.0%
		Present	Count	1	2	3
			% of Total	5.0%	10.0%	15.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Total	Before	Absent	Count	44	0	44
			% of Total	73.3%	0.0%	73.3%
		Present	Count	8	8	16
			% of Total	13.3%	13.3%	26.7%
	Total		Count	52	8	60
			% of Total	86.7%	13.3%	100.0%

Salmonella Drinkers Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	11	0	11
			% of Total	55.0%	0.0%	55.0%
		Present	Count	5	4	9
			% of Total	25.0%	20.0%	45.0%
	Total		Count	16	4	20
			% of Total	80.0%	20.0%	100.0%
Cycle2	Before	Absent	Count	13	0	13
			% of Total	65.0%	0.0%	65.0%
		Present	Count	4	3	7
			% of Total	20.0%	15.0%	35.0%
	Total		Count	17	3	20
			% of Total	85.0%	15.0%	100.0%
Cycle3	Before	Absent	Count	19		19
			% of Total	95.0%		95.0%
		Present	Count	1		1
			% of Total	5.0%		5.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	43	0	43
			% of Total	71.7%	0.0%	71.7%
		Present	Count	10	7	17
			% of Total	16.7%	11.7%	28.3%
	Total		Count	53	7	60
			% of Total	88.3%	11.7%	100.0%

Salmonella Feeders Cross-tabulation

Cycle No				After		
				Absent	Present	Total
Cycle1	Before	Absent	Count	13	0	13
			% of Total	65.0%	0.0%	65.0%
		Present	Count	5	2	7
			% of Total	25.0%	10.0%	35.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle2	Before	Absent	Count	17	0	17
			% of Total	85.0%	0.0%	85.0%
		Present	Count	2	1	3
			% of Total	10.0%	5.0%	15.0%
	Total		Count	19	1	20
			% of Total	95.0%	5.0%	100.0%
Cycle3	Before	Absent	Count	20		20
			% of Total	100.0%		100.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	50	0	50
			% of Total	83.3%	0.0%	83.3%
		Present	Count	7	3	10
			% of Total	11.7%	5.0%	16.7%
	Total		Count	57	3	60
			% of Total	95.0%	5.0%	100.0%

Salmonella Feed Loaders Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	6	0	6
			% of Total	50.0%	0.0%	50.0%
		Present	Count	4	2	6
			% of Total	33.3%	16.7%	50.0%
	Total		Count	10	2	12
			% of Total	83.3%	16.7%	100.0%
Cycle2	Before	Absent	Count	11		11
			% of Total	91.7%		91.7%
		Present	Count	1		1
			% of Total	8.3%		8.3%
	Total		Count	12		12
			% of Total	100.0%		100.0%
Cycle3	Before	Absent	Count	11		11
			% of Total	91.7%		91.7%
		Present	Count	1		1
			% of Total	8.3%		8.3%
	Total		Count	12		12
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	28	0	28
			% of Total	77.8%	0.0%	77.8%
		Present	Count	6	2	8
			% of Total	16.7%	5.6%	22.2%
	Total		Count	34	2	36
			% of Total	94.4%	5.6%	100.0%

Salmonella Fans Cross-tabulation

Cycle No				After Absent	Total
Cycle1	Before	Absent	Count	11	11
			% of Total	68.8%	68.8%
		Present	Count	5	5
			% of Total	31.3%	31.3%
		Total	Count	16	16
			% of Total	100.0%	100.0%
Cycle2	Before	Absent	Count	15	15
			% of Total	93.8%	93.8%
		Present	Count	1	1
			% of Total	6.3%	6.3%
		Total	Count	16	16
			% of Total	100.0%	100.0%
Cycle3	Before	Absent	Count	13	13
			% of Total	81.3%	81.3%
		Present	Count	3	3
			% of Total	18.8%	18.8%
		Total	Count	16	16
			% of Total	100.0%	100.0%
Total	Before	Absent	Count	39	39
			% of Total	81.3%	81.3%
		Present	Count	9	9
			% of Total	18.8%	18.8%
		Total	Count	48	48
			% of Total	100.0%	100.0%

Salmonella Vents Cross-tabulation

Cycle No				After		
				Absent	Present	Total
Cycle1	Before	Absent	Count	9	1	10
			% of Total	45.0%	5.0%	50.0%
		Present	Count	6	4	10
			% of Total	30.0%	20.0%	50.0%
	Total		Count	15	5	20
			% of Total	75.0%	25.0%	100.0%
Cycle2	Before	Absent	Count	17	0	17
			% of Total	85.0%	0.0%	85.0%
		Present	Count	1	2	3
			% of Total	5.0%	10.0%	15.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle3	Before	Absent	Count	20		20
			% of Total	100.0%		100.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	46	1	47
			% of Total	76.7%	1.7%	78.3%
		Present	Count	7	6	13
			% of Total	11.7%	10.0%	21.7%
	Total		Count	53	7	60
			% of Total	88.3%	11.7%	100.0%

Campylobacter Annexe Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	11		11
			% of Total	68.8%		68.8%
		Present	Count	5		5
			% of Total	31.3%		31.3%
	Total		Count	16		16
			% of Total	100.0%		100.0%
Cycle2	Before	Absent	Count	12	0	12
			% of Total	75.0%	0.0%	75.0%
		Present	Count	3	1	4
			% of Total	18.8%	6.3%	25.0%
	Total		Count	15	1	16
			% of Total	93.8%	6.3%	100.0%
Cycle3	Before	Absent	Count	15	0	15
			% of Total	93.8%	0.0%	93.8%
		Present	Count	0	1	1
			% of Total	0.0%	6.3%	6.3%
	Total		Count	15	1	16
			% of Total	93.8%	6.3%	100.0%
Total	Before	Absent	Count	38	0	38
			% of Total	79.2%	0.0%	79.2%
		Present	Count	8	2	10
			% of Total	16.7%	4.2%	20.8%
	Total		Count	46	2	48
			% of Total	95.8%	4.2%	100.0%

Campylobacter Crevices Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	13	0	13
			% of Total	65.0%	0.0%	65.0%
		Present	Count	1	6	7
			% of Total	5.0%	30.0%	35.0%
	Total		Count	14	6	20
			% of Total	70.0%	30.0%	100.0%
Cycle2	Before	Absent	Count	18	0	18
			% of Total	90.0%	0.0%	90.0%
		Present	Count	1	1	2
			% of Total	5.0%	5.0%	10.0%
	Total		Count	19	1	20
			% of Total	95.0%	5.0%	100.0%
Cycle3	Before	Absent	Count	14	0	14
			% of Total	70.0%	0.0%	70.0%
		Present	Count	4	2	6
			% of Total	20.0%	10.0%	30.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Total	Before	Absent	Count	45	0	45
			% of Total	75.0%	0.0%	75.0%
		Present	Count	6	9	15
			% of Total	10.0%	15.0%	25.0%
	Total		Count	51	9	60
			% of Total	85.0%	15.0%	100.0%

***Campylobacter* Drinkers Cross-tabulation**

Cycle No				After		
				Absent	Present	Total
Cycle1	Before	Absent	Count	10	0	10
			% of Total	50.0%	0.0%	50.0%
		Present	Count	4	6	10
			% of Total	20.0%	30.0%	50.0%
	Total		Count	14	6	20
			% of Total	70.0%	30.0%	100.0%
Cycle2	Before	Absent	Count	10	0	10
			% of Total	50.0%	0.0%	50.0%
		Present	Count	8	2	10
			% of Total	40.0%	10.0%	50.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle3	Before	Absent	Count	17		17
			% of Total	85.0%		85.0%
		Present	Count	3		3
			% of Total	15.0%		15.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	37	0	37
			% of Total	61.7%	0.0%	61.7%
		Present	Count	15	8	23
			% of Total	25.0%	13.3%	38.3%
	Total		Count	52	8	60
			% of Total	86.7%	13.3%	100.0%

Campylobacter Feeders Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	12	0	12
			% of Total	60.0%	0.0%	60.0%
		Present	Count	6	2	8
			% of Total	30.0%	10.0%	40.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle2	Before	Absent	Count	16	0	16
			% of Total	80.0%	0.0%	80.0%
		Present	Count	2	2	4
			% of Total	10.0%	10.0%	20.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle3	Before	Absent	Count	20		20
			% of Total	100.0%		100.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	48	0	48
			% of Total	80.0%	0.0%	80.0%
		Present	Count	8	4	12
			% of Total	13.3%	6.7%	20.0%
	Total		Count	56	4	60
			% of Total	93.3%	6.7%	100.0%

Campylobacter Feed Loader Cross-tabulation

Cycle No				After		
				Absent	Present	Total
Cycle1	Before	Absent	Count	6	0	6
			% of Total	50.0%	0.0%	50.0%
		Present	Count	5	1	6
			% of Total	41.7%	8.3%	50.0%
	Total		Count	11	1	12
			% of Total	91.7%	8.3%	100.0%
Cycle2	Before	Absent	Count	11	0	11
			% of Total	91.7%	0.0%	91.7%
		Present	Count	0	1	1
			% of Total	0.0%	8.3%	8.3%
	Total		Count	11	1	12
			% of Total	91.7%	8.3%	100.0%
Cycle3	Before	Absent	Count	10	0	10
			% of Total	83.3%	0.0%	83.3%
		Present	Count	1	1	2
			% of Total	8.3%	8.3%	16.7%
	Total		Count	11	1	12
			% of Total	91.7%	8.3%	100.0%
Total	Before	Absent	Count	27	0	27
			% of Total	75.0%	0.0%	75.0%
		Present	Count	6	3	9
			% of Total	16.7%	8.3%	25.0%
	Total		Count	33	3	36
			% of Total	91.7%	8.3%	100.0%

Campylobacter Fans Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	10		10
			% of Total	62.5%		62.5%
		Present	Count	6		6
			% of Total	37.5%		37.5%
		Total	Count	16		16
			% of Total	100.0%		100.0%
Cycle2	Before	Absent	Count	13	0	13
			% of Total	81.3%	0.0%	81.3%
		Present	Count	1	2	3
			% of Total	6.3%	12.5%	18.8%
		Total	Count	14	2	16
			% of Total	87.5%	12.5%	100.0%
Cycle3	Before	Absent	Count	13		13
			% of Total	81.3%		81.3%
		Present	Count	3		3
			% of Total	18.8%		18.8%
		Total	Count	16		16
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	36	0	36
			% of Total	75.0%	0.0%	75.0%
		Present	Count	10	2	12
			% of Total	20.8%	4.2%	25.0%
		Total	Count	46	2	48
			% of Total	95.8%	4.2%	100.0%

Campylobacter Vents Cross-tabulation

Cycle No				After		Total
				Absent	Present	
Cycle1	Before	Absent	Count	8	1	9
			% of Total	40.0%	5.0%	45.0%
		Present	Count	6	5	11
			% of Total	30.0%	25.0%	55.0%
	Total		Count	14	6	20
			% of Total	70.0%	30.0%	100.0%
Cycle2	Before	Absent	Count	17	0	17
			% of Total	85.0%	0.0%	85.0%
		Present	Count	1	2	3
			% of Total	5.0%	10.0%	15.0%
	Total		Count	18	2	20
			% of Total	90.0%	10.0%	100.0%
Cycle3	Before	Absent	Count	20		20
			% of Total	100.0%		100.0%
	Total		Count	20		20
			% of Total	100.0%		100.0%
Total	Before	Absent	Count	45	1	46
			% of Total	75.0%	1.7%	76.7%
		Present	Count	7	7	14
			% of Total	11.7%	11.7%	23.3%
	Total		Count	52	8	60
			% of Total	86.7%	13.3%	100.0%

Appendix T: Multiplex qPCR quantification of *Salmonella* spp. and *Campylobacter* spp. on swab samples collected on each location

Table T1: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected at the annex, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	5/16 (31.3)	5/16 (31.3)	3.43 (3.27; 3.69)	3.17 (2.67; 3.67)
	AD	ND	ND	ND	ND
2	BC	3/16 (18.8)	4/16 (25)	3.79 (3.53; 4.1)	3.78 (3.15; 4.39)
	AD	ND	1/16 (6.3)	ND	2.97 (2.97)
3	BC	1/16 (6.3)	1/16 (6.3)	3.91 (3.91)	4.44 (4.44)
	AD	1/16 (6.3)	1/16 (6.3)	3.47 (3.47)	2.96 (2.96)
Total	BC	9/48 (18.8)	10/48 (20.8)	3.61 (3.27; 4.1)	3.54 (2.67; 4.44)
	AD	1/48 (2.1)	2/48 (4.2)	3.47 (3.47)	2.97 (2.96; 2.97)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T2: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on crevices, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	12/20 (60)	7/20 (35)	3.45 (3.26; 4.08)	3.34 (3.07; 3.56)
	AD	6/20 (30)	6/20 (30)	3.34 (2; 4.49)	2.93 (2.3; 3.33)
2	BC	1/20 (5)	2/20 (10)	4.89 (4.89)	3.87 (3.61; 4.12)
	AD	ND	1/20 (5)	ND	2.69 (2.69)
3	BC	3/20 (15)	6/20 (30)	3.59 (3.45; 3.68)	3.34 (2.55; 4.01)
	AD	2/20 (10)	2/20 (10)	3.55 (3.44; 3.66)	2.79 (2.55; 3.02)
Total	BC	16/60 (26.7)	15/60 (25)	3.57 (3.26; 4.89)	3.41 (2.55; 4.12)
	AD	8/60 (13.3)	9/60 (15)	3.39 (2; 4.49)	2.87 (2.3; 3.33)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T3: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on drinkers, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	9/20 (45)	10/20 (50)	3.8 (3.23; 4.43)	3.85 (2.43; 4.72)
	AD	4/20 (20)	6/20 (30)	3.05 (2; 3.59)	2.69 (2.37; 3.02)
2	BC	7/20 (35)	10/20 (50)	3.56 (3.37; 3.73)	3.48 (2.79; 4.24)
	AD	3/20 (15)	2/20 (10)	3.32 (3.17; 3.45)	3.21 (3.2; 3.22)
3	BC	1/20 (5)	3/20 (15)	3.25 (3.25)	3.32 (2.48; 4.51)
	AD	ND	ND	ND	ND
Total	BC	17/60 (28.3)	23/60 (38.3)	3.67 (3.23; 4.43)	3.62 (2.43; 4.72)
	AD	7/60 (11.7)	8/60 (13.3)	3.17 (2; 3.59)	2.82 (2.37; 3.22)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T4: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on feed loaders, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	6/12 (50)	6/12 (50)	3.36 (3.08; 3.62)	3.22 (2;3.8)
	AD	2/16 (16.7)	1/12 (8.3)	3.49 (3.18; 3.8)	2.46 (2.46)
2	BC	1/12 (8.3)	1/12 (8.3)	4.35 (4.35)	2.87 (2.87)
	AD	ND	1/12 (8.3)	ND	2.66 (2.66)
3	BC	1/12 (8.3)	2/12 (16.7)	3.82 (3.82)	3.47 (2.84; 4.12)
	AD	ND	1/12 (8.3)	ND	2.36 (2.36)
Total	BC	8/36 (22.2)	9/36 (25)	3.57 (3.08; 4.35)	3.24 (2; 4.12)
	AD	2/36 (5.6)	3/36 (8.3)	3.49 (3.18; 3.8)	2.49 (2.36; 2.66)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T5: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on feeders, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	7/20 (35)	8/20 (40)	3.6 (3.15; 4.01)	3.44 (2.76; 4.16)
	AD	2/20 (10)	2/20 (10)	3.17 (2.95; 3.4)	2.41 (2.28; 2.53)
2	BC	3/20 (15)	4/20 (20)	3.58 (3.5; 3.73)	3.52 (2.88; 4.11)
	AD	1/20 (5)	2/20 (10)	3.47 (3.47)	2.28 (2.23; 2.33)
3	BC	ND	ND	ND	ND
	AD	ND	ND	ND	ND
Total	BC	10/60 (16.7)	12/60 (20)	3.6 (3.15; 4.01)	3.46 (2.76; 4.16)
	AD	3/60 (5)	4/60 (6.7)	3.27 (2.95; 3.47)	2.34 (2.23;2.53)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T6: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on fans, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	5/16 (31.3)	6/16 (37.5)	3.27 (2.64; 3.96)	3.08 (3.35; 4.21)
	AD	ND	ND	ND	ND
2	BC	1/16 (6.3)	3/16 (18.8)	3.8 (3.8)	3.38 (2.54; 4.66)
	AD	ND	2/16 (12.5)	ND	2.77 (2.63; 2.91)
3	BC	3/16 (18.8)	3/16 (18.8)	3.8 (3.45; 4.3)	4.29 (4.16; 4.4)
	AD	ND	ND	ND	ND
Total	BC	9/48 (18.8)	12/48 (25)	3.50 (2.64; 4.3)	3.82 (2.54; 4.66)
	AD	ND	2/48 (4.2)	ND	2.77 (2.63; 2.91)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.

Table T7: Mean prevalence and microbial counts of *Salmonella* spp. *and Campylobacter* spp. of samples collected on vents, analysed by qPCR

Cycle	Stage of Cleaning	No. positive/ total no. samples (%)		Mean (minimum; maximum) Log_{10} CFU/mL of positive samples	
		Salmonella	*Campylobacter*	*Salmonella*	*Campylobacter*
1	BC	10/20 (50)	11/20 (55)	3.57 (3.27; 4)	4.01 (3.37; 4.64)
	AD	5/20 (25)	6/20 (30)	3.02 (2; 3.54)	3.52 (2.99; 3.82)
2	BC	3/20 (15)	3/20 (15)	3.64 (3.56; 3.73)	3.69 (3.58; 3.9)
	AD	2/20 (10)	2/20 (10)	3.27 (3.07; 3.48)	3.75 (3.72; 3.77)
3	BC	ND	ND	ND	ND
	AD	ND	ND	ND	ND
Total	BC	13/60 (21.7)	14/60 (23.3)	3.59 (3.27; 4)	3.93 (3.37; 4.64)
	AD	7/20 (11.7)	8/60 (13.3)	3.09 (2; 3.54)	3.57 (2.99;3.82)

Note: Numbers are means of samples collected on four sheds combined during three consecutive cycles of cleaning regimes. ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime comprising of pre-washing, washing, and disinfection.