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

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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

Intensive poultry production due to public demand raises the risk of contamination, creating potential foodborne hazards to consumers. The prevalence and microbial load of pathogens *Campylobacter*, *Salmonella*, *S. aureus* and *E. coli* was determined by standard methods at the farm level. After disinfection, swab samples collected on wall crevices, drinkers and vents were heavily contaminated, as accumulated organic matter and dusts were likely protecting pathogens against the disinfectants used. The annex floor also showed high microbial concentrations, suggesting the introduction of pathogens from the external environments, highlighting the importance of erecting hygiene barriers at the entrance of the main shed. Therefore, pathogen control measures and proper application of disinfectants are recommended as intervention strategies. Additionally, qPCR was evaluated as a quantification tool. qPCR showed limitations with samples containing low microbial counts due to the low detection limit of the method. Thus, bacterial pre-enrichment of test samples may be necessary to improve the detection of pathogens by qPCR.

Keywords

Foodborne pathogens, broiler farm, cleaning and disinfection, contamination.
Introduction

Poultry is extensively produced in broiler farms and is processed industrially to meet public demand. However, this type of food production gives an opportunity for the presence of foodborne pathogens and their toxins, thereby posing risks to consumers (Sanders 1999). There are approximately 250 foodborne diseases that can cause various symptoms such as nausea, abdominal cramping, diarrhea, and vomiting (MPI n.d). Among these diseases, the most common are caused by strains of Salmonella, Campylobacter, Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) (Lopez et al. 2016).

Salmonella, Campylobacter, E. coli and S. aureus can infect poultry through vertical and/or horizontal transmission. For instance, S. Enteritidis can invade the bird host defence mechanism, and transmit infection vertically to its progeny before eggshell formation (Cox et al. 2000; Fine 1975; Foley et al. 2011; Gantois et al. 2009). Previous studies also suggested that E. coli is transmitted vertically due to its inability to survive outside the host environment (Winfield and Groisman 2003). Nonetheless, horizontal transmission is the most likely cause of pathogen outbreaks in broiler farms. S. Typhimurium, Campylobacter, E. coli and S. aureus can be transmitted horizontally in poultry, due to faecal droppings and residual presence of pathogens from the previous flock. Pest, flies, and beetles as pathogen vectors can also be problematic because they can amplify existing contamination by coming into contact with inaccessible areas of the shed, as well as contaminating feed and water sources (Beutin 2006; Davies and Breslin...
The occurrence of food-related infections from poultry requires better control interventions and improved prevention strategies, particularly at the farm level (Callejon et al. 2015). Different areas of a broiler shed can harbour 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 the broiler shed due to contaminated faeces that are excreted from infected birds (MPI 2001). Meanwhile, *E. coli* can be isolated from soil and water, only when they mimic conditions found in the internal mucosa (Beutin 2006). Whereas, *S. aureus* is mainly found in areas that are difficult to clean and sanitise (MPI 2001). Several bio-security risks have been identified in the New Zealand poultry industry, and some mitigation strategies have also been established (Geale et al. 2006).

Microbiological analysis has been an important part of microbial safety management in the food chain (Chapela et al. 2015; Chen et al. 2012). Monitoring and controlling foodborne pathogens are traditionally carried out using standard methods that are based on culture-dependent techniques, including standard biochemical characterisations. However, rapid methods such as PCR and quantitative PCR (qPCR) have become important quantification tools in several fields of biological research due to their ability to rapidly detect, identify and quantify pathogens or beneficial bacteria (Postollec et al. 2011). In this study, standard methods and multiplex qPCR were performed to determine
the prevalence and microbial load of contaminating *Campylobacter, Salmonella, E. coli* and *S. aureus* from different locations of four broiler sheds, before cleaning and after disinfection.
Materials and Methods

Background

Swab samples were collected from four broiler sheds at a poultry farm in Auckland, New Zealand to determine the efficiency of cleaning and disinfection after the removal of grown birds. The efficiency of cleaning and disinfection protocols between the six-week growth cycle were evaluated three times, from March 2016 to July 2016.

The cleaning and disinfection regimes performed comprised of pre-cleaning, cleaning and disinfection. Pre-cleaning was performed after the removal of used soft wood shavings (litter) where the operator used a blower (14.0 m³/minute; Back-Pack Blower BBX7600, Makita Japan), to blow dust and debris out of the shed from all surfaces. The cleaning step commenced with water blasting (27.6 k-pascals; GX390 High-Pressure Water Blaster, Honda USA) all surfaces with Biostrip 3000™ pre-disinfectant solution (Chemetall New Zealand Limited) to remove organic soils, oil/fat, and residual biofilms that would affect the performance of the main disinfectant. Disinfection was then carried out by applying Glutasan QCT (Chemetall New Zealand Limited) to all surfaces of the shed at high pressure (27.6 k-pascals; GX390 High-Pressure Water Blaster, Honda USA) and air-dried for 24 hours. Once air-dried, 50 mm of clean soft wood shavings were placed on the floor and the whole shed was fumigated with Glutasen ULV (Chemetall New Zealand Limited).

The annex (which housed records, foot bath, temperature and humidity controllers) was cleaned separately from the main shed. The floors, walls, ceiling and controllers of the
annex were cleaned and disinfected with 3 % Triton QCT HF (Chemetall New Zealand Limited).

**Sampling plan**

A total of 248 swab samples were collected before and after disinfection. The swabs comprised of samples from the annex floor (n = 32), fans (n = 32), vents (n = 40), feed loaders (n = 24), feeders (n = 40), drinkers (n = 40), and wall crevices (n = 40). The sample size for each location was determined by using a software called Win Episcope® (Thrusfield et al. 2001), based on an estimated prevalence of 50 % with 95 % confidence interval (Castro-Hermida et al. 2015; Kich et al. 2011; Vanantwerpen et al. 2014).

Win Episcope® was also used to design a simple random sampling plan for each location that was swabbed. The random numbers obtained were used to generate codes for feeders, feed loaders, drinkers, fans, vents, and the annex floor, which were then 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 of the crevices that had been sampled, wall crevices located on one area of the shed were swabbed.

**Sample Collection and Preparation**

The wet and dry swab sampling protocol of ISO 18593 was used to collect the samples (ISO 2004). Each set of the wet and dry sample swabs was prepared according to...
Andrews and Hammack (2003). Nine mL peptone water (0.1 %, Fort Richard NZ) were aseptically added into each swab sample that contained one mL of buffered peptone water (BPW). The swab sample suspension (x10\(^1\)) was mixed thoroughly (30 s) with a vortex mixer (VM-96B, JEIO TECH Korea), then analysed for *Salmonella*, *Campylobacter*, *S. aureus* and *E. coli*, using standard methods and multiplex qPCR analysis.

**Bacterial Stock Cultures**

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

**Standard Methods**

*Isolation of Salmonella and Campylobacter*

The isolation of *Salmonella* was carried out following the procedure of Andrews et al. (2007). Five mL of the swab sample suspension (x10\(^1\)) were pre-enriched with 45 mL of BPW, and incubated at 35 °C for 24 hours. Positive and negative control samples were also included in parallel with the swab samples. Of the pre-enriched samples, 0.1 mL was aseptically transferred into 10 mL pre-warmed Rappaport Vassiliadis Soya (RVS) broth tubes (Fort Richard NZ), and incubated at 42 °C for 24 hours. Ten µL of the enriched sample were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Fort Richard NZ).
and Modified Brilliant Green (MBGA) agar (Fort Richard NZ) plates. The prepared plates were incubated for 24 hours at 35 °C. Suspected colonies of *Salmonella* on each agar were confirmed with latex agglutination tests (Microgen® *Salmonella* Rapid Latex Test Kit; Ngaio Diagnostics NZ).

The isolation of *Campylobacter* was performed according to Hunt et al. (2001). One mL of the swab sample suspension (x10^1) was enriched with 25 mL Boltons Broth (Fort Richard NZ). Positive and negative control samples were also included in parallel with the swab samples. The prepared samples were incubated under microaerophilic conditions (84 % N\textsubscript{2}, 10 % CO\textsubscript{2}, 6 % O\textsubscript{2}) for four hours at 35 °C, and then 44 hours at 42 °C. Ten µL of the enriched sample were then streaked onto Modified Charcoal-Cefoperazone-Deoxycholate (mCCDA) plates (Fort Richard NZ). The prepared plates were incubated under microaerophilic conditions at 42 °C for 48 hours. Suspected *Campylobacter* colonies found on each plate were confirmed with the oxidase (Oxoid™ Oxidase Strips; Fort Richard NZ) and latex agglutination tests (Microgen® *Campylobacter* Rapid Latex Test Kit; Ngaio Diagnostics NZ).

*Enumeration of S. aureus and E. coli using 3M Petrifilm™*

Serial dilutions (up to x10^5) of each swab sample suspension (x10^1) were plated on 3M Petrifilm™ plates (3M™ USA), for the enumeration of *S. aureus* and *E. coli*. The enumeration of *S. aureus* was conducted following the AOAC Official Method 2003.11 (Wendy et al. 2003), using Petrifilm™ Staph Express Count Plates (3M™ USA). Meanwhile, the enumeration of *E. coli* was based on the AOAC Official Method 991.14.
(AOAC 1998), using Petrifilm™ *E. coli* Coliform Count plate (3M™ USA; Schraft and Watterworth 2005; Warnes and Keevil 2004).

**Quantitative PCR**

**Primers and Probes**

The primer sequences used in this study were based on the report by Cremonesi et al. (2014) with the following target genes: tetrathionate reductase response regulator (ttrR) gene located within *Salmonella* pathogenicity islands; 16S rRNA of *Campylobacter*; and the heat-shock protein gene (htrA) of *S. aureus*, respectively (Table 1). The oligonucleotide probes were prepared with reporter dyes on the 5’ end, and a non-fluorescent quencher (NFQ) on the 3’ end. *Salmonella* probes were labelled with the reporter dye FAM™ (6-carboxyfluorescein; Applied Biosystems® USA), whereas *Campylobacter* and *S. aureus* probes were labelled with VIC® (Applied Biosystems® USA).

**Extraction and Purification of DNA**

Bacterial genomic DNA was extracted from one mL of stock culture and test samples using the Qiagen DNeasy® Blood and Tissue Kit (Bio-Strategy NZ) as described by the manufacturer. After extraction, the DNA was eluted twice to increase the DNA yield of the sample. Once eluted, DNA samples were stored at 4 °C in a refrigerator for short term storage (one week), or in a -20 °C freezer (Fisher and Pykel NZ) for longer term storage (one year).
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 were added to the mixture containing 10 µL of Taqman® Gene Expression Master Mix (2x; Applied Biosystems® USA), one µL of primer (10 µM; Applied Biosystems® USA), one µL of probe (5 µM; Applied Biosystems® USA), and milli-Q water.

The reaction volume of 20 µL was also used for multiplex qPCR reactions. Four µL of DNA samples/standards were added to the mixture containing 10 µL of Taqman® Gene Expression Master Mix (2x), one µL of each primer (10 µM), and one µL of each probe (5 µM).

All qPCR assays were performed with the StepOne™ Real-Time PCR System (Applied Biosystems® USA). The amplification was carried out at 50 °C for two minutes to activate the 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.

Validation of multiplex qPCR using different concentrations of DNA

Experiments with ten-fold serial dilutions from DNA stocks were conducted to determine the accuracy of the multiplex qPCR assay. Firstly, DNA control stocks were prepared from pure cultures. The concentration and purity of DNA were determined spectrophotometrically (ASP 370 Micro-volume UV/Vis spectrophotometer, ACTGene
USA) at an absorbance of 260 nm and 260/280 nm, respectively. The DNA control stocks were adjusted to 50 ng/µL with Tris-EDTA (TE) buffer. Ten-fold serial dilutions were performed on the 50 ng/µL working stock to obtain a dilution of x10⁵. The 10-fold serial dilutions (x10⁵ – x10¹) corresponded to 1 to 10,000 pg respectively, when two µL of stock dilution were added to a 20 µL qPCR reaction.

In the multiplex qPCR reaction, the DNA concentration of one target was kept constant at 10,000 pg, whilst the other target had varying DNA concentrations, which ranged from 1 pg to 10,000 pg (Eckford-Soper and Daugbjerg 2015).

Preparation of the DNA standard curve for the qPCR analysis of environmental samples

Standard curves for qPCR were constructed using inoculated environmental samples. Bacterial dilutions (x10¹ - x10⁷) of pure cultures were prepared and spread-plated onto solidified nutrient agar (Fort Richard NZ) plates for *Salmonella* / *S. aureus* and Columbia Sheep Blood Agar (Fort Richard NZ) plates for *Campylobacter*, to determine the bacterial concentration of each diluted stock culture. One mL of each diluted stock culture was inoculated into environmental samples that had been confirmed to be *Salmonella*, *Campylobacter* and *S. aureus* negative, as determined by standard methods (Eckford-Soper and Daugbjerg 2015).

Sample suspensions were set aside for DNA extraction, which was conducted in triplicate to account for any possible loss during DNA extraction. These extractions were then analysed by using the multiplex qPCR reaction conditions.
Comparing the efficiency of plate count and qPCR method

Environmental samples (n=100) were analysed to compare the efficiency of the standard method and then qPCR method. The qPCR standard curve generated from inoculated environmental samples as previously described, were used to calculate the concentration (Log\(_{10}\) CFU/mL) of *S. aureus* from C\(_t\) values (Macé et al. 2013). The analysis of samples by qPCR were carried out in triplicate, and the mean values were calculated.

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 each location for each sampling time was determined by cross-tabulation. All bacterial cell counts were transformed into Log\(_{10}\) CFU/mL and reported as mean values. The detection limit of the bacterial enumeration was 1 Log\(_{10}\) CFU/mL. To visualise the data, the data were presented as stacked bar graphs.

qPCR 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 et al. 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 = \([10 \cdot (-1/slope) – 1]\). In this study, the efficiency between 80 % - 115 % was considered acceptable (Callbeck et al. 2013; Zhang and Fang 2006).
To estimate the concentration (Log\(_{10}\) CFU/mL) of an unknown sample from the qPCR assay, the equation \( Y = 10^{((X - c)/m)} \times 10 \) 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 derived from qPCR analysis, and \( m \) = slope for \( X \) (Hu et al. 2012; Kephart and Bushon 2009; Yang et al. 2003).

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 scatterplot with an identity line (\( y = x \)) was also used to determine the agreement between the methods.

**Results and Discussion**

Poultry has been implicated in majority of food poisoning incidences worldwide (Sanders 1999). Previous studies reported that the prevalence of pathogens in bird and environmental samples at the farm level were correlated with the prevalence observed in carcass rinse from the same flocks during processing (Berghaus et al. 2013; Schroeder et al. 2014; Volkova et al. 2010). Consequently, effective management practices on the farm are essential to reduce contamination during processing at the factory and of the final products.

In this study, three cleaning and disinfection regimes were evaluated during three, six-week growth cycles, from March 2016 to July 2016 in the autumn – winter season of New
Zealand. A total of 248 environmental swab samples were collected from different areas (annex floor, wall crevices, plastic drinkers, metal feed loaders, plastic feeder, fans, and vents) of four sheds, before and after the cleaning and disinfection regimes, respectively. Due to the nature of the field trials, uncontrolled variables were expected. The uncontrolled variables may include heterogeneity of contamination levels (Luyckx et al. 2015; Newell and Fearnley 2003), seasonal variability of pathogen prevalence (Allen and Newell 2005; Evans and Sayers 2000; Refregier-Petton et al. 2001), and different cleaning teams operating inconsistent practices (Refregier-Petton et al. 2001).

Detection of pathogens using standard methods

Annex floor

In poultry rearing, pathogen control measures are an integral part of farming operations aimed at preventing infection of livestock (Allen and Newell 2005). As reported in previous studies, human activity can easily introduce pathogens into the farm shed, which may infect the flocks (Allen and Newell 2005; Guerin et al. 2007; Marin et al. 2011). Therefore, hygiene barriers are important, not only for the farmers, but also for other personnel such as farm workers, contract cleaners, visitors and inspectors (Robyn et al. 2015).

An annex shed is a facility that allows farm workers to sanitise their hands and use footbaths before tending to the birds (Allen and Newell 2005). Although several hygiene parameters have been reported (Allen and Newell 2005; Evans and Sayers 2000; McDowell et al. 2008), the sanitation of the annex shed has not been previously investigated.
Prior to cleaning, the prevalence of *Salmonella* (Fig. 1) and *Campylobacter* (Fig. 2) in samples obtained from the annex floor steadily decreased between cycles 1, 2, and 3. The steady decrease of the prevalence of the bacteria counts between each cycle may be caused by seasonal variability, especially because the mean daily temperatures from March 2016 to July 2016 decreased from 27 °C to 17 °C (Metservice NZ n.d) respectively. Previous studies have reported that flock infection peaked in the summer and autumn months, compared to winter and spring (Lara and Rostagno 2013; McDowell et al. 2008; Refregier-Petton et al. 2001). Vandeplas et al. (2010) reported a seasonal effect of *Campylobacter* prevalence of 33.3 % and 100 % during the winter and summer months, respectively. Meanwhile, Schulz et al. (2011) reported no significant seasonal effect on the prevalence of *Salmonella*, but the study concluded that seasonal variations could be due to an existing relationship between temperature and pathogen transmission. In this study, *Salmonella* was not detected in cycles 1 and 2, but was detected in cycle 3 after disinfection (Fig. 1), whereas *Campylobacter* was detected in cycles 2 and 3 after disinfection with 6.3 % (1/16) prevalence in both cycles (Fig. 2). These results suggested that the cleaning regime of the annex floor effectively decreased the prevalence of *Salmonella* and *Campylobacter*.

Viable counts of *S. aureus* and *E. coli* were also investigated in the annex floor (Table 2). The mean cell counts for *S. aureus* decreased after disinfection in cycles 1, 2, and 3 with 1.27, 2.47, and 1.49 Log$_{10}$ CFU/mL, respectively. Meanwhile, the cleaning regime reduced *E. coli* cell counts in cycles 1 and 2 by 1.17 and 0.53 Log$_{10}$ CFU/mL respectively.
The reductions of *S. aureus* and *E. coli* cell counts after disinfection were not significant (p > 0.05) to enable us to determine the efficiency of cleaning. The presence of high cell counts before cleaning could have affected the efficacy of the disinfectant used in this study (Ray and Bhunia 2007). The increase of *E. coli* cell counts (1.63 Log\(_{10}\) CFU/mL) in cycle 3 after disinfection may have been caused by cross contamination by farm workers, which suggested the importance of hygiene barriers at the entrance of the main farm shed. Previous studies have reported that footwear worn by workers at the farm may be possible sources of contamination, especially when the footwear had contact with soil and animal excreta. This may be applicable to the results of most pathogens, and not only for *E. coli* (Friese et al. 2013; Herman et al. 2003; Locking et al. 2001; Rose et al. 2000).

**Wall crevices**

Organic matter can accumulate in crevices which can protect pathogens from chemical agents and contribute to residual contamination or biofilm formation (Allen and Newell 2005). Most broiler sheds are made of concrete, and crevices can form when they are not properly maintained (Allen and Newell 2005). It is therefore important that the shed is properly cleaned to remove organic materials before disinfection (Mueller-Doblies et al. 2010). Poorly disinfected crevices can be improved by spot treatment with double strength disinfection (Davies et al. 2001). In addition, the risk of flock infection can be reduced by covering crevices (Luyckx et al. 2015).

Compared to the prevalence of *Salmonella* (Fig. 1) observed on wall crevices before cleaning, *Campylobacter* (Fig. 2) was lower in cycle 1, whilst cycles 2 and 3 had higher
prevalence of the bacterium. After disinfection, the prevalence of *Salmonella* and *Campylobacter* in wall crevices were similar to cycles 1 and 3 at 30% (6/20) and 10% (2/20), respectively. However, in cycle 2, the prevalence of *Campylobacter* was 5% (1/20), whilst *Salmonella* was not detected by the methods used. The high prevalence of *Salmonella* and *Campylobacter* observed after disinfection in cycle 1 may be caused by organic residues that was not removed during cleaning. Mueller-Doblies et al. (2010) reported similar results during their study involving floor cracks. When organic matter or biofilms persist after power-washing, the strength of the disinfectant used can be reduced, resulting in higher bacterial counts. High bacterial concentration observed before cleaning in cycle 1 could have been caused by the inefficient application of the disinfectant used in the previous cycle.

Cell counts of *S. aureus* in samples from wall crevices decreased after disinfection compared to *E. coli* counts (Table 2). In cycles 1, 2 and 3 after disinfection, *S. aureus* cell counts decreased by 1.5, 0.65 and 2 Log_{10} CFU/mL, respectively. Meanwhile, *E. coli* cell counts only 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 *S. aureus* and *E. coli* cell counts obtained on wall crevices after disinfection, were not significant (p > 0.05). The low efficiency of the cleaning and sanitising agent may be due to the presence of organic residues on wall crevices. Another factor is that excess water remaining on wall crevices after washing may have diluted the disinfectant, thereby reducing the efficacy of the chemical used.
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 entire water system to reduce the risk of microbial colonisation and biofilm formation (Allen and Newell 2005; Cox and Pavic 2010; Evans and Sayers 2000; Luyckx et al. 2015).

Before cleaning, the prevalence of *Salmonella* (Fig. 1) and *Campylobacter* (Fig. 2) observed on drinkers in cycle 3 were 5 % (1/20) and 15 % (3/20). Whereas, in cycles 1 and 2, the prevalence of *Campylobacter* was 50 % (10/20) on both cycles, and the prevalence of *Salmonella* was 45 % (9/20) and 35 % (7/20), respectively. After disinfection, *Salmonella* (Fig. 1) and *Campylobacter* (Fig. 2) were not detected in cycle 3, indicating that the cleaning and disinfection regime was efficient, especially when low bacterial counts are present before cleaning. Since drinkers are frequently contaminated by organic matter during rearing, low bacterial prevalence on drinkers before cleaning were not frequently observed (Poppe et al. 1991). For instance, the high prevalence of *Salmonella* and *Campylobacter* after disinfection in cycles 1 and 2 on drinkers may be explained by the presence of residual organic matter containing pathogens. As reported by Battersby et al. (2017), ineffective cleaning and disinfection may be caused by a range of factors including ineffective concentrations of disinfectants, poor hygiene design of equipment, failure to remove organic matter prior to disinfection, using inefficient procedures, short contact time and recontamination.
Viable cell counts of *E. coli* on drinkers before cleaning were 1 to 2 Logs lower than the mean counts of *S. aureus* (Table 2). After disinfection, cell counts of *S. aureus* decreased by 1.46 (cycle 1), 0.7 (cycle 2), and 1.59 (cycle 3) Log_{10} CFU/mL. Meanwhile, *E. coli* was only present in cycles 1 and 2, which decreased by 2.05 and 0.81 Log_{10} CFU/mL, respectively. Despite having standard cleaning and disinfection protocols, the microbial log reductions after cleaning were not consistent, which may be explained by discrepancies of cleaning standards by different cleaning teams. Additionally, the sensitivity and efficacy of the disinfectant used is dependent on the bacterial concentration before cleaning. In this regard, since *E. coli* cell counts were lower before cleaning (Table 2), the mean counts of *E. coli* had a higher Log_{10} decrease after performing disinfection, compared to the mean counts of *S. aureus*. Bower and Daeschel (1999) reported similar results and concluded that the penetration of disinfectants was less effective when aggregation of bacterial cells are present.

**Feed loaders and feeders**

Feed serves as an indirect route of bacterial transmission to poultry flocks (Maciorowski et al. 2007). Several studies on pathogen contamination routes of feed reported that feed contamination can be caused by contaminated dust when it becomes airborne, or by carry-over contamination from the previous rearing of birds (Friese et al. 2013; Maciorowski et al. 2007; Marin et al. 2011). In this study, contamination of feeders and feed loaders by pathogens were further investigated. Feed infection is often associated with surface contamination, especially since feed undergoes extensive treatments
(chemical and heat) to control pathogen colonisation (Friese et al. 2013; Maciorowski et al. 2007; Marin et al. 2011; Doyle and Erickson 2006).

In this study, feed loaders were expected to have low prevalence of *Salmonella* (Fig. 1) and *Campylobacter* (Fig. 2), because feed loaders are positioned at a higher location (one meter above the floor). Therefore, birds had limited access to the feed loaders. This assumption agreed with the low prevalence observed in cycles 2 and 3 before cleaning. However, in cycle 1, both *Salmonella* and *Campylobacter* had a prevalence of 50% (6/12). The high bacterial prevalence may be attributed to contaminated dust that settled in feed loaders during rearing (Chinivasagam et al. 2009). After disinfection, *Salmonella* was not detected in cycles 2 and 3, but was observed in cycle 1, with 16.7% (2/12) prevalence (Fig. 1). Meanwhile, the prevalence of *Campylobacter* was 8.3% (1/12) in all three cycles (Fig. 2). Davies and Wray (1996) reported similar results and concluded that pathogen re-contamination on disinfected feed loaders attributes to inefficient cleaning from the previous cleaning regime.

In this study, feeders had higher prevalence before cleaning, compared to feed loaders. Since birds can easily access feeders, contamination by pathogens could have been introduced by dust, dirty litter, regurgitation of feed, or the transfer of infection by beaks (Herman et al. 2003; Marin et al. 2011). After disinfection, the presence of *Salmonella* and *Campylobacter* on feeders could lead to the infection of birds. Residual feed left on feeders and/or improperly applied disinfectants were possible reasons for re-contamination. During sampling, it was observed that feeders were not inverted to allow ‘drip drying’. To
reduce carry-over infection to the next flock, it is important to dry shed equipment and
shed surfaces thoroughly (Allen and Newell 2005).

Comparing cell counts of *S. aureus* and *E. coli* before cleaning and after disinfection
(Table 2), mean counts of *S. aureus* decreased by 1.51 (cycle 1), 2.9 (cycle 2), and 2.48
(cycle 3) Log$_{10}$ CFU/mL on feed loaders; and 2.15 (cycle 1), 2.01 (cycle 1), and 2.93 (cycle
1) Log$_{10}$ CFU/mL on feeders. Meanwhile, *E. coli* was not detected in cycle 3 from both
locations, but the pathogen was present in cycles 1 and 2, with mean reductions 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 (Table 2). The reductions of *S. aureus* on feed loaders and feeders were
comparatively higher than of *E. coli* after disinfection. This outcome was unexpected
because *E. coli* (Gram negative) has a thinner peptidoglycan layer compared to *S. aureus*
(Gram positive) (Mahalanabis et al. 2009). Therefore, sanitizers and disinfectants were
expected to easily penetrate *E. coli* cells causing them to shrivel and die (Mahalanabis et
al. 2009). The lower reductions of *S. aureus* and *E. coli* on feed loaders compared to
feeders may have been caused by the variability in cleaning standards of different
(cleaning) teams. Feed loaders were expected to have higher reductions in cell counts
because they are constructed from metal which are easier to clean and disinfect due to
their smoother surfaces (Poppe et al. 1991).

**Fan and vents**

Over the years, producers have installed modern ventilation systems. Fans and vents are
important features of broiler sheds, as it manages humidity and temperature during
poultry production (Appleby et al. 2004; Aviagen 2009; Broom and Fraser 2015; Calvet et al. 2011; Mench et al. 2008). The facilities are also used to control the build-up of ammonia gas from poultry waste to reduce incidences of respiratory diseases (Sonaiya and Swan 2007). Large volumes of air are moved through the shed by a negative pressure, providing optimum conditions for broiler growth (Aviagen 2009; Chinivasagam et al. 2009; Mench et al. 2008). However, the air may contain a range of bacteria from the external and/or internal environmental sources of the shed. Therefore, efficient disinfection of the fan ventilation system is vital to reduce the chances of pathogens being airborne, especially when dust is present (Chinivasagam et al. 2009).

Compared to the prevalence of Salmonella (Fig. 1) on fans before cleaning, Campylobacter counts (Fig. 2) were higher in cycles 1 and 2 at 37.5 % (6/16) and 18.8 % (3/16), respectively. As a result, Salmonella was not present in all three cycles after disinfection (Fig. 1), whereas Campylobacter was present in cycle 2 at 12.5 % (2/16) (Fig. 2). The efficiency of the cleaning regime applied on fans could be associated with the reduction of pathogen prevalence. However, since Campylobacter was only detected in cycle 2 after disinfection, it was suspected that the results could be linked to some inconsistencies in the cleaning regimes.

The prevalence of Salmonella (Fig. 1) and Campylobacter (Fig. 2) on vents before cleaning were at their highest levels in cycle 1, which were 50 % (10/20) and 55 % (11/20), respectively. After disinfection, the high prevalence of Salmonella (Fig. 1) and Campylobacter (Fig. 2) could be explained by the accumulated dust trapped between the

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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 dust into feed and drinking water which may be hazardous to flocks (Chinivasagam et al. 2009).

The high prevalence of *Salmonella* and *Campylobacter* on the fan ventilation system in cycle 1 could be explained by seasonal variations in poultry production. In the warmer months, high ventilation is achieved by ensuring that all fans are fully operational, and vents are fully opened to control odor and moisture during rearing (Aviagen 2009; Mench et al. 2008; Wabeck 2002). In low temperature months, vents are partially opened to reduce draught, as well as allow cool air to mix with the air in the shed before coming into contact with the birds (Aviagen 2009; Mench et al. 2008; Vest and Tyson 1991). 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 have suggested that open ventilation during the warmer months can encourage flies to act as vectors for pathogens, leading to the spread of infection among flocks (McDowell et al. 2008; Newell and Fearnley 2003; Vandeplas et al. 2010).

Before cleaning, *S. aureus* counts on fans and vents were not significantly (p < 0.05) different between cycles 1, 2 and 3, whereas *E. coli* counts decreased by 1 Log$_{10}$ between the cleaning cycles (Table 2). The steady decrease of *E. coli* before cleaning may be caused by seasonal variation. Comparing the cell counts before cleaning and after
disinfection, the mean cell counts of *S. aureus* in cycles 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. Meanwhile, *E. coli* counts decreased by 1.44 and 0.14 on fans, and 1.85 and 0.97 on vents in cycles 1 and 2, respectively. The higher mean counts of *S. aureus* obtained after disinfection compared to *E. coli* may suggest that *S. aureus* was less sensitive to the concentration of the disinfectant used in this study.

**Enumeration of bacteria using multiplex qPCR**

Microbiological assays are used regularly to monitor potential microbial contamination and risks (Rodríguez-Lázaro 2013). However, qPCR has emerged as an important microbial quantification tool due to its ability to rapidly detect segments of the DNA that are unique to the species and strain (WHO 2003). qPCR methods are now used to detect and quantify *E. coli* O157 in raw milk to support investigations in food safety programs (Paul et al. 2013), *Campylobacter* 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).

**Sensitivity of multiplex qPCR on the analysis of variable concentrations of target pathogens**

Bacteria can co-exist at different concentrations in samples (Van Giau et al. 2016). Therefore, the ability of multiplex qPCR assay to amplify multiple DNA targets in different concentrations is important. No assay inhibition was observed when *Campylobacter* and *Salmonella* DNA targets were amplified, especially when *Campylobacter* was present at
high DNA concentration (10,000 pg). However, when *Salmonella* was present at high DNA concentration (10,000 pg), the DNA amplification of *Campylobacter* decreased from one to 100 pg (Table 3). Previous studies have reported similar results, where the detection of a target DNA was inhibited when the other target was present at a higher concentration (Dai, Peng, Chen, Cheng and Wu 2013; Hyeon et al. 2010; Wang, Li and Mustapha 2007). Similarly, if the other target was present in moderate or low concentration, the detection of the target DNA is not inhibited (Dai et al. 2013; Hyeon et al. 2010; Wang et al. 2007). This suggests that the high concentration of one target DNA can interfere with the DNA amplification of a target at low concentration in multiplex qPCR. This leads to increased $C_t$ values, resulting in reduced assay sensitivity (Dai et al. 2013).

**Validation of qPCR assay for the analysis of environmental samples**

In environmental and food microbiology, qPCR standard curves are used to quantify target DNA concentrations in diverse samples (Brankatschk et al. 2012). Biological and food samples contain inhibitory substances that are not found in standard samples prepared from purified DNA 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). Typical examples of inhibitors include dead biomass and soil containing humic and fulvic acids; sludge containing fats, proteins, polyphenols, heavy metals; waste-water containing polysaccharides, metal ions, RNases; and food containing fats, glycogen, polysaccharides, minerals and enzymes (Schrader et al. 2012).

The standard curves used for environmental and food analysis are often based on
CFU/mL. Leblanc-Maridor et al. (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* negative samples with 10-fold dilutions of *Campylobacter* suspensions of each reference strain. The study concluded that the established qPCR assay was highly specific despite the presence of PCR inhibitors in the sample.

In this study, the multiplex qPCR assay of inoculated *Salmonella* and *Campylobacter* target in environmental samples were species-specific. In Fig. 3, *Salmonella* \( (y = -3.2459x + 46.315, \ R^2 = 0.99) \) and *Campylobacter* \( (y = -3.6953x + 45.815 \ with \ R^2 = 0.99) \) DNA amplification had a linear relationship between C\text{t} and CFU/mL, with an amplification efficiency of 103.27 % and 86.47 %. The detection limit of each assay was 3.24 - 8.24 Log\textsubscript{10} CFU/mL for *Salmonella*, and 2.97 - 7.97 Log\textsubscript{10} CFU/mL for *Campylobacter*.

**Comparison between standard method and qPCR**

The agreement between the standard method and qPCR demonstrated the validity of the quantification data produced by the latter (Clais et al. 2015). In this study, the plate counts of *S. aureus* (Log\textsubscript{10} CFU/mL) in environmental samples were comparable to the estimated qPCR data (Log\textsubscript{10} CFU/mL). The quantified samples analysed by qPCR were calculated by generating standard curves using data from inoculated samples. The linear equation \( (y = -3.6701x + 47.456) \) from the standard curve was used to calculate the estimated Log\textsubscript{10} CFU/mL of each sample (Fig. 4).
The data obtained from both methods were expected to lie within the identity line and to be normally distributed (Clais et al. 2015). In this study, samples quantified by plate counts and qPCR ranged from 1 to 6.1 Log10 CFU/mL, and 3.5 to 6 Log10 CFU/mL, respectively. Fig. 5 shows an agreement between the two methods only when the bacterial concentration was above 3.5 Log10 CFU/mL. Below this concentration, the agreement between the methods diminished because the points in the scatter plot did not lie across the identity line (Bland and Altman 1995). qPCR equivalents had Log10 counts of 3 – 4 Log10 CFU/mL when the plate counts were 1 – 2 Log10 CFU/mL. Similar results were reported from previous studies, in which qPCR estimates were higher than the results of the plate count method (Botaro et al. 2013; Botteldoorn et al. 2008; De Carvalho et al. 2015; Hein et al. 2006). The high cell concentration of qPCR estimates may be attributed to several factors such as the presence of DNA from dead cells; the presence of viable but non-culturable cells due to the adaptation of cells to environmental stress; and a single CFU on a plate may be generated by more than one cell (Postollec et al. 2011).

The Bland-Altman plot between the results of the plate count and qPCR method was evaluated to assess the difference between the two methods. Fig. 6 shows a mean difference of -0.79 Log10 CFU/mL between plate count and qPCR methods, with a 95% confidence interval (limits of agreement) of 0.90 to -2.47 Log10 CFU/mL. However, most of the 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 showed a slight agreement between the two methods, suggesting that qPCR enumeration
may be used to estimate the bacterial concentration of samples. However, it is important to note the limitations of qPCR, especially when low bacterial counts are anticipated.

**Enumeration of Salmonella and Campylobacter using multiplex qPCR**

Environmental samples that indicate the presence of *Salmonella* and *Campylobacter* using standard methods were enumerated using multiplex qPCR. The estimated qPCR counts of *Salmonella* and *Campylobacter* from each location are summarised in Table 4. Before cleaning, the estimated mean counts of *Salmonella* and *Campylobacter* 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* was not detected in the samples collected from the fans. Comparing the results before cleaning and after disinfection, *Salmonella* counts had the lowest Log$_{10}$ decrease in feed loaders (0.08 Log$_{10}$ CFU/mL), followed by the annex floor (0.14 Log$_{10}$ CFU/mL), wall 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*, *Campylobacter* was detected in samples from fans after disinfection, with a reduction of 1.05 Log$_{10}$ CFU/mL. Additionally, feeders had the highest cell count reduction (1.12 Log$_{10}$ CFU/mL) after disinfection, followed by the annex floor (0.57 Log$_{10}$ CFU/mL), drinkers (0.8 Log$_{10}$ CFU/mL), wall 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 when analysed by qPCR.

qPCR can accurately determine cell counts if the microbial concentration of the sample are within the detection limit of the assay (Rothrock et al. 2009; Ruijter et al. 2009).
study, the microbial counts generated by qPCR after disinfection were below the detection limit of the assay. This result may be attributed to poor DNA yield from the microorganisms, probably due to the low initial bacterial concentration (Postollec et al. 2011). A pre-enrichment step of the test sample has been suggested to increase the DNA yield, which may improve the efficiency of the method (De Boer et al. 2015; De Oliveira et al. 2010). 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 $2.6 \times 10^4$ CFU/g$^{-1}$ to $< 10$ CFU/g$^{-1}$. Josefsen et al. (2004) reported similar results during the enumeration of low *Campylobacter* in chicken rinse samples by including a 20 hour enrichment step under standard conditions before qPCR analysis. The enrichment step together with the qPCR method dilutes potential inhibitory substances and create favourable conditions for the growth of target bacterial pathogens to detectable numbers (Ibekwe and Grieve 2003; Joyner et al. 2014).

**Conclusions**

*Salmonella, Campylobacter, S. aureus* and *E. coli* were present in swab samples obtained pre-cleaning and post-disinfection during the cleaning regimes. Before cleaning, the four pathogens were present at high prevalence and concentrations from all seven swabbed locations. Of the swabbed locations in the broiler sheds, wall crevices and drinkers were the most contaminated after disinfection, probably due to the presence of residual organic matter that remained after cleaning. Therefore, multiplex qPCR is an important tool for monitoring potential contamination trends and microbial risk because of
its ability to detect, identify and quantify multiple pathogens in one assay. The Bland-Altman plot of difference showed agreement between plate count method and qPCR, but with limitations. Samples collected before cleaning can be quantified by qPCR due to high bacterial load, whereas the bacterial concentration post-disinfection was below the detection limit of the qPCR assay used. Therefore, bacterial pre-enrichment of test samples may be recommended to improve the quantification of *Salmonella* and *Campylobacter* by qPCR.

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quantification of microorganisms in environmental samples. Applied Microbiology 
and Biotechnology. 70(3): 281-289.
**Figure Legends**

Fig. 1. Prevalence of *Salmonella* spp. in sampled locations during three consecutive cycles of cleaning regimes. Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime including pre-washing, washing and disinfection.

Fig. 2. Prevalence of *Campylobacter* spp. in sampled locations during three consecutive cycles of cleaning regimes. Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime including pre-washing, washing and disinfection.

Fig. 3. Standard curves of inoculated samples *Salmonella* spp. and *Campylobacter* spp. Note: Serial dilutions of each standard ranged from 2 – 9 Log10 CFU/mL per reaction. Efficiency (E) was calculated based on the slope determined by linear regression analysis. Slope and R2 were calculated based on average results from triplicate data sets of both DNA extraction and qPCR analysis.

Fig. 4. *S. aureus* standard curves from inoculated samples. Note: Serial dilutions of each standard ranged from 2 – 9 Log10 CFU/mL per reaction. Efficiency (E) was calculated based on the slope determined by linear regression analysis. Slope and R2 were calculated based on the average results from triplicate data sets of both DNA extraction and qPCR analysis.

Fig. 5. Enumeration of *S. aureus* (Log10 CFU/mL) obtained by qPCR, which is plotted against the enumeration obtained by plate count method (Log10 CFU/mL) on an identity line (y=x).

Fig. 6. Bland-Altman analysis between qPCR and plate count methods. Note: Bland-Altman plot represents the average of the enumeration results (Log10 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)).
Table 1. Set of Primers and probes for each microorganism

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Target gene</th>
<th>Sequences (5’-3’)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella</strong></td>
<td>ttrR F</td>
<td>CGAAGAGACCCCTGTCGTACT</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>ttrR R</td>
<td>AAGTGGACGCATCGACCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ttrR P</td>
<td>6FAM-TCGCCGTCGCTATT-</td>
<td>MGBNFQ</td>
</tr>
<tr>
<td><strong>Campylobacter</strong></td>
<td>16s rRNA F</td>
<td>CGCCGCGTGGGAGGAT</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>16s rRNA R</td>
<td>GGTACCCTCAGAATTCTCTTCCAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16s rRNA P</td>
<td>VIC-ACGCTCCGAAAAGTTYG-T-MGBNFQ</td>
<td></td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>htrA F</td>
<td>GAAAGTAATATCAGACAAATCAAATAC</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>htrA R</td>
<td>AGTACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>htrA P</td>
<td>TCTTCCGTTAAGTTAATGGCTTCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIC-CAGATTCCGACAATTTT-MGBNFQ</td>
<td></td>
</tr>
</tbody>
</table>

Note: F = Forward Primer; R = Reverse Primer; MGBNFO = Minor Groove Binder Non Florescent Quencher. Source: Cremonesi et al. (2014)
Table 2. Mean counts of *S. aureus* and *E. coli* in sampled locations during 3 cycles of the cleaning regimes

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean (SD) Log$_{10}$ CFU/ml of positive samples</th>
<th>S. aureus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 2</td>
<td>Cycle 3</td>
</tr>
<tr>
<td>Annex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>4.92</td>
<td>5.68</td>
<td>4.36</td>
</tr>
<tr>
<td>AD</td>
<td>(0.48)</td>
<td>(0.66)</td>
<td>(0.55)</td>
</tr>
<tr>
<td>Crevices</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>5.27</td>
<td>6.02</td>
<td>5.15</td>
</tr>
<tr>
<td>AD</td>
<td>(1.05)</td>
<td>(1.81)</td>
<td>(0.92)</td>
</tr>
<tr>
<td>Drinkers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>3.77</td>
<td>5.37</td>
<td>3.15</td>
</tr>
<tr>
<td>AD</td>
<td>(1.34)</td>
<td>(1.18)</td>
<td>(0.83)</td>
</tr>
<tr>
<td>Feed Loader</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>6.20</td>
<td>5.22</td>
<td>3.93</td>
</tr>
<tr>
<td>AD</td>
<td>(0.60)</td>
<td>(1.29)</td>
<td>(0.92)</td>
</tr>
<tr>
<td>Feeders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>4.74</td>
<td>4.52</td>
<td>2.34</td>
</tr>
<tr>
<td>AD</td>
<td>(0.80)</td>
<td>(0.80)</td>
<td>(1.24)</td>
</tr>
<tr>
<td>Fans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>5.77</td>
<td>5.93</td>
<td>4.78</td>
</tr>
<tr>
<td>AD</td>
<td>(0.65)</td>
<td>(1.11)</td>
<td>(0.83)</td>
</tr>
<tr>
<td>Vents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>3.85</td>
<td>3.24</td>
<td>2.33</td>
</tr>
<tr>
<td>AD</td>
<td>(0.86)</td>
<td>(1.00)</td>
<td>(0.58)</td>
</tr>
</tbody>
</table>
| Note: NA = not applicable because only one sample was positive; ND = not detected; BC = before cleaning; AD = after disinfection; Cycle = cleaning regime (pre-washing, washing, disinfection)
Table 3. Evaluating the sensitivity of duplex reactions based on different bacterial concentration

<table>
<thead>
<tr>
<th></th>
<th>Singleplex</th>
<th>Multiplex</th>
<th>$C_t$ Difference</th>
<th>SD of $C_t$ Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>10,000-pg</td>
<td>19.44</td>
<td>19.39</td>
<td>0.05</td>
</tr>
<tr>
<td>S</td>
<td>10,000-pg</td>
<td>19.98</td>
<td>20.00</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>1,000-pg</td>
<td>23.57</td>
<td>23.47</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>100-pg</td>
<td>27.16</td>
<td>26.88</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>10-pg</td>
<td>30.35</td>
<td>30.30</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>1-pg</td>
<td>33.78</td>
<td>33.63</td>
<td>0.15</td>
</tr>
<tr>
<td>S</td>
<td>10,000-pg</td>
<td>19.98</td>
<td>20.02</td>
<td>0.32</td>
</tr>
<tr>
<td>C</td>
<td>10,000-pg</td>
<td>19.44</td>
<td>19.12</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>1,000-pg</td>
<td>22.78</td>
<td>21.91</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>100-pg</td>
<td>26.31</td>
<td>24.20</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>10-pg</td>
<td>29.73</td>
<td>27.31</td>
<td>2.42</td>
</tr>
<tr>
<td></td>
<td>1-pg</td>
<td>33.42</td>
<td>29.68</td>
<td>3.74</td>
</tr>
</tbody>
</table>

Note: $C_t$ values are based on mean triplicates results for Salmonella spp. (S) and Campylobacter spp. (C) DNA amplification; SD = standard deviation.
Table 4. Mean counts of *Salmonella* spp. and *Campylobacter* spp. analysed by multiplex qPCR

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

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.
Fig. 1. Prevalence of *Salmonella* spp. in sampled locations during three consecutive cycles of cleaning regimes.

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime including pre-washing, washing and disinfection.
Fig. 2. Prevalence of *Campylobacter* spp. in sampled locations during three consecutive cycles of cleaning regimes.

Note: BC = before cleaning; AD = after disinfection; Cycle = cleaning regime including pre-washing, washing and disinfection.
Fig. 3. 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.
Fig. 4. *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.
Fig. 5. Enumeration of *S. aureus* (Log_{10} CFU/mL) obtained by qPCR, which is plotted against the enumeration obtained by plate count method (Log_{10} CFU/mL) on an identity line (y=x).
Fig. 6. 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)).