Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations

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Abstract

Poultry meat consumption in New Zealand has been increasing since 1975 with the highest peak reported in 2006. The total poultry meat consumption was 36.5 kg per capita in the year ending September 2006. Consumption of contaminated food with raw poultry can lead to campylobacteriosis, which is a food-borne disease that causes gastroenteritis in humans and it is a major problem in New Zealand. There were 12,776 reported cases of campylobacteriosis in 2007, which accounts for 65.9% of the overall notified diseases. Campylobacteriosis can lead to Guillain-Barré syndrome in some patients, an autoimmune disorder of the peripheral nervous system. Campylobacteriosis is caused by consumption of either *Campylobacter jejuni* or *Campylobacter coli*. *Campylobacter* spp. have been found in commercially raised poultry being infected predominantly by *C. jejuni*. *C. jejuni* has been found associated with biofilms of other bacterial species in the watering supplies and plumbing systems of animal husbandry facilities and animal-processing plants. A biofilm is an assemblage of microbial cells that is associated with a surface and the cells are enclosed in a matrix of polysaccharides, which provides a survival advantage to the bacteria in the film. In this study, the ability to form biofilm was measured in a laboratory assay using microtitre plates. *C. jejuni* strains in monoculture were shown to attach to the abiotic surface and form biofilms to various degrees, thus potentially enhancing their survivability in the poultry environment. *C. jejuni* was also shown to have the ability to attach and survive in mixed-microbial populations. Biofilm formation may play a role in the epidemiology of *C. jejuni* infections. *Enterococcus faecalis* and *Staphylococcus simulans* may play a role in the biofilm formation in the poultry environment as both of these microorganisms were able to form, and harbour *C. jejuni* in their biofilms. *Pseudomonas aeruginosa* seemed to inhibit biofilm formation and *C. jejuni* in the mixed-microbial population. Further studies are required to establish control measures against the formation of biofilms containing *C. jejuni* in poultry processing plants and farms in New Zealand to reduce the reservoir of contamination and thus reduce the incidence of campylobacteriosis.

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Chapter 1 Background to *Campylobacter* jejuni in poultry industry and New Zealand

Poultry meat consumption in New Zealand has been increasing since 1975 with the highest peak reported in 2006 (103). The total poultry meat consumption was 36.5 kg per capita in the year ending September 2006. Poultry meat was the highest of total meat consumption in the year ending September 2006 (125). The percentage of tonnes of poultry meat consumed was 35.32% followed by beef and veal. The lowest percentage of tonnes of meat consumed was mutton with 2.99%. The percentage of tonnes of meat consumed is shown in Figure 1.

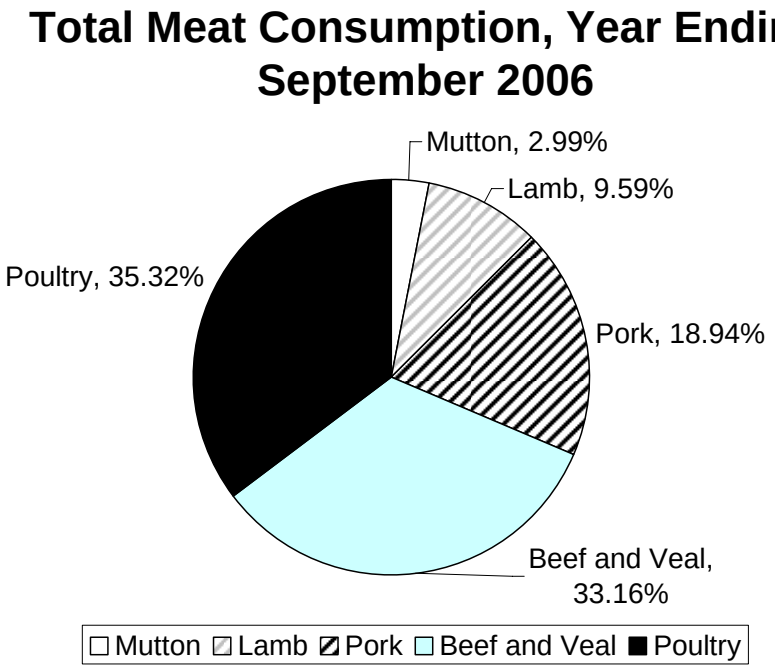

Figure 1 The percentage of total meat consumed, year ending September 2006.
Source: www.pianz.org.nz

The increased poultry meat consumption may be due to the change of diet in New Zealand. Poultry meat is a good source of protein. It is leaner and easier to digest when compared to other red meat. Besides that, poultry meat is cheaper than other meats as the turnover rate in the poultry farm is faster than other meat-producing farms. The life span of a broiler (chicken that is breed for meat production) from a one-day old chick to its ideal weight, is about 35 - 42 days.

Poultry production has been increasing steadily over the years. The lowest poultry meat production was in 1993 with 73,299 tonnes compared with 2005 when 160,433 tonnes of poultry meat was produced (125). In 2007, 149,868 tonnes of poultry meat was produced in New Zealand, in which, 144,318 tonnes of this was chicken meat constituting 96.3% of all poultry meat production (24). This may be due to the increase in the demand of poultry meat as well as better farming management in the poultry industry. The feed conversion rate (FCR) of poultry has been improving steadily over the years. The FCR is used to determine the amount of feed required for a kg weight gain.

Consumption of undercooked poultry meat can lead to campylobacteriosis, which is a food-borne disease that causes gastroenteritis in humans. It is a major problem in New Zealand as it has the highest level of notifications of food-borne diseases compared to other food-borne diseases. Campylobacteriosis does not usually result in death, however, there was one death reported in 2007 (121).

A sequel of campylobacteriosis is Guillain-Barré syndrome (GBS). Guillain-Barré syndrome is an autoimmune disorder of the peripheral nervous system. The *Campylobacter jejuni* strains can be transformed into a number of potential GBS-inducible transformants, which exhibit a high degree of genetic and phenotypic diversity that leads to Guillain-Barré syndrome (158). Campylobacteriosis is caused by consumption of either *C. jejuni* or *C. coli* (51). *C. jejuni* has been found in poultry farm and poultry processing plant (2, 3, 20, 52, 56, 82, 142, 155).

New Zealand has two to three times higher rates of campylobacteriosis than other developed countries, and more than ten times higher than United States of America (11). There were 15,873 reported cases of campylobacteriosis in 2006 compared with 1335 reported cases of salmonellosis. Campylobacteriosis accounted for 68.4% of all notified diseases in New Zealand in 2006 (120). Campylobacteriosis was still the highest notified disease in 2007, comprising 65.9% of all notifications (121).

The rates of campylobacteriosis cases have increased steadily in New Zealand since the disease first became notifiable in 1980. The rate of campylobacteriosis per 100,000 population increased by 41% between 2001 and 2006. However, the rate of campylobacteriosis has been decreasing steadily in the year 2008. The lowest reported rate was 201.7 cases per 100,000 people in June 2008. It has dropped about 44.3% compared with the same time last year (125).

Changes in reporting behaviour by doctors are unlikely to have contributed to the increased rates of campylobacteriosis as they have a fairly high level of notification of gastrointestinal diseases that are laboratory-confirmed (12, 165). The pattern of campylobacteriosis notifications in New Zealand is not markedly different from that observed overseas (90).

Although campylobacteriosis is highly seasonal, with a marked peak in most summers and a winter trough, the seasonality peak in New Zealand is different from several countries in Europe as it is less consistent from year to year, and the peak is more prolonged (11, 109, 119). Campylobacteriosis is more commonly reported in New Zealand males and the highest age specific rate occurred in children aged 1-4 years, followed by the 20-29 year age group in 2006 and 2007(120, 121).

Cases of campylobacteriosis have been estimated to cost NZ$40,136,000 annually, 73% of the total economic cost of food-borne infectious intestinal disease in New Zealand (137). This estimate included direct and indirect medical costs, the value of productive days lost, and the statistical value of mortality, but not the value of lost quality of life.

Campylobacter species can be further differentiated into definitive types by multi locus sequence typing (MLST). The most common MLST of *C. jejuni* in the world is MLST-45 and MLST-48. However, MLST-474 and MLST-190 of *C. jejuni* cause most of campylobacteriosis in New Zealand (58). These MLSTs are unique as they are rarely found internationally. In addition, they have been isolated from poultry products in the Manawatu region.

Chapter 2 Literature review – *Campylobacter jejuni* in the poultry industry

2.1 General introduction

Campylobacter jejuni belongs to the family *Campylobacteraceae.* The family *Campylobacteraceae* includes the genera *Campylobacter, Arcobacter, Sulfurospirillum,* and the generically misclassified *Bacteroides ureolyticus.* All are Gram negative, nonsaccharolytic bacteria with microaerophilic growth requirements and a low G+C content (105). *Campylobacter* usually exist as commensals or parasites in human or domestic animals, while *Sulfurospirillum* exist as free-living environmental bacteria. *Arcobacter* can exist as commensals, parasites, or free-living environmental bacteria.

Campylobacter is mostly slender, spirally curved rod, motile, thermophilic, and microaerophilic microorganisms that do not form spores. *Campylobacter* cells are usually 0.2 to 0.8 μm wide and 0.5 to 5 μm long (105). Aged cells of *Campylobacter* or cells that are subjected to environmental stress usually appear as spherical or coccoid bodies. This is considered as degenerative form rather than dormant stage of the cell. Most of the cells have a single polar unsheathed flagellum at one or both end of the cells. This flagellum gives the *Campylobacter* the characteristic screw-like motion. However, some of the species are either non-motile (*Campylobacter gracilis*) or have multiple flagella (*Campylobacter showae*).

Most of the *Campylobacter* grow under microaerophilic conditions and have a respiratory and chemoorganotrophic type of metabolism, while some can grow under aerobic or anaerobic conditions. *Campylobacter* use amino acids and intermediates of the tri-carboxylic acid cycle as energy. *Campylobacter* do not ferment or oxidise carbohydrate. Typical biochemical reactions are the reduction of fumarate to succinate; a negative methyl red reaction and acetoin and indole production; and for most species, reduction of nitrate, absence of hippurate hydrolysis, and presence of oxidase activity (105). The optimum growth temperature for *Campylobacter* is between 30 to 37°C. However, the optimum growth temperature for *Campylobacter jejuni* is between 42 to 45°C. *C. jejuni* is still able to survive outside the optimum temperature even at low temperature as low as 4°C (23, 27, 36, 154).

2.2 History

Campylobacter spp. was classified as *Vibrio* spp. until 1973. This was because *Campylobacter* spp. was not thought to be a cause of diarrhoea in humans until 1957. *Vibrio fetus* infection caused abortion in sheep and cattle. Later, it was classified as *Campylobacter fetus.* In 1947, *V. fetus* caused an abortion in a woman, and during the next three decades, the organism was believed to be a rare, opportunistic, invasive pathogen. By mid-to-late 1980s, *Campylobacter* spp. was determined as one of the most common causes of human gastrointestinal diseases (4).

2.3 Taxonomy and pathogenicity

Campylobacter consists of 16 species, two other species have recently been described (55, 79, 112). The species and the subspecies are shown in the Table 1.

Table 1 The 16 species of *Campylobacter* and its subspecies and biovar

Genus	Species	Subspecies (subsp.)	Biovar (bv.)
Campylobacter	*canadensis*		
Campylobacter	*coli*		
Campylobacter	*concisus*		
Campylobacter	*curvus*		
Campylobacter	*fetus*	*fetus*	
		venerealis	
Campylobacter	*gracilis*		
Campylobacter	*helveticus*		
Campylobacter	*hominis*		
Campylobacter	*hyointestinalis*	*hyointestinalis*	
		lawsonii	
Campylobacter	*insulaenigrae*		
Campylobacter	*jejuni*	*doylei*	
		jejuni	
Campylobacter	*lanienae*		
Campylobacter	*lari*		
Campylobacter	*mucosalis*		
Campylobacter	*rectus*		
Campylobacter	*showae*		
Campylobacter	*sputorum*		bubulus
			sputorum
			paraureolyticus
Campylobacter	*upsaliensis*		

Campylobacter canadensis is a recently discovered species that has been found in the bacterial floral of the cloacae of whooping cranes in Canada. The prevalence of *C. canadensis* and its role are at present unknown (79). The cells are polymorphic in shape (sigmoid to coccoid), 0.5 – 0.6 μm wide and 0.7 – 2.6 μm long. The motility of the cells is controlled by a single polar flagellum.

Campylobacter coli is pathogenic to humans and is one of the most important human enteropathogens among *Campylobacter* spp. The biochemical characteristics are almost similar to *C. jejuni;* the only difference is that *C. coli* does not have the ability to hydrolyse hippurate.

Campylobacter concisus is a heterogeneous species that has been confirmed by a wide range of molecular techniques; AFLP analysis revealed at least four distinct genonospecies within *C. concisus* (105). This species only grows in the presence of hydrogen in a microaerophilic environment. Hydrogen is used as an electron source. Although there is a high prevalence of *C. concisus* in human diseases, the role as a human enteric pathogen remains unclear.

Campylobacter curvus was originally described as *Wolinella curva*. The prevalence of *C. curvus* in humans is extremely low. There is evidence that *C. curvus* is the etiologic agent of bloody diarrhea and Brainerd's diarrhea, however, it cannot be conclusively proven (1). Besides that, C. *curvus* has been isolated from patients with either Guillain-Barré or Fisher's syndrome but it does not play a role in the development of these neurological diseases (89).

Campylobacter fetus has two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp *veneralis,* both of which are pathogens. *C. fetus* subsp. *fetus* is usually associated with abortions in sheep and cattle; however, it is uncommon in humans. *C. fetus* subsp. *veneralis* is a causative agent of bovine genital campylobacteriosis, which is mainly associated with bovine genital tract (105).

Campylobacter gracilis is an oxidase-negative *Campylobacter* species. The cell morphology is a straight rod and aflagellate. The pathogenicity of *C. gracillis* is usually associated with serious tissue infection and high antibiotic resistance, however, its role as a pathogen is believed to be underestimated as there is a lack of conclusive evidence of pathogenicity (105).

Campylobacter helveticus a non-human isolate, is usually isolated from domestic animals such as felines and canines (105). *C. helveticus* is catalase-negative and has a relative DNA homolog with thermophilic *Campylobacter* species, notably *Campylobacter upsaliensis.*

Campylobacter hominis was previously described as "*Candidatus Campylobacter hominis*." Cells of *C. hominis* are straight, blunt-ended bacilli or coccobacilli, and are non-motile. Although the cells are non-motile, certain strains of *C. hominis* produce numerous irregular fimbriae-like structures (93). *C. hominis* can only be cultured under anaerobic conditions.

Campylobacter hyointestinalis is commonly associated with pigs, and occasionally found in humans. This species is further subdivided into *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii. C. hyointestinalis* subsp. *hyointestinalis* and is isolated from pigs with the intestinal disorder, porcine proliferative enteritis. The pathogenic role of *C. hyointestinalis* subsp. *hyointestinalis* in humans is uncertain. *C. hyointestinalis* subsp. *lawsonii* has been isolated from the stomach of pigs, and its clinical significance is unknown (105).

Campylobacter lari has been isolated from the intestinal contents of seagulls, river water, shellfish, and occasionally from human. The pathogenicity of this species is unknown. This species is resistant to nalidixic acid. However, nalidixic acid susceptible strains has been identified as *C. lari* variants by one-dimensional whole-cell protein electrophoresis and semiquantitative DNA-DNA hybridization (105).

Campylobacter insulaenigrae is another recently described species. It has been isolated from marine mammals in Scotland and California (105). The role of this species may be restricted to marine mammals, even though it is genetically distinct from *C. lari*. Stoddard *et al*., (2007) suggest that C. *insulaernigrae* may have evolved from or at least shares a common ancestor with *C. lari* (146).

Campylobacter lanienae cells are slender, slightly curved rods, with single, bipolar unsheathed flagella. *C. lanienae* was originally isolated from two abattoir workers during a routine screening of abattoir workers. Since then *C. lanienae* has been isolated from swine and bovine faecal samples using conventional and quantitative PCR (80, 105, 135).

Campylobacter mucosalis has been isolated from the intestinal mucosa of pigs and from the porcine oral cavity. It is originally classified as *Campylobacter sputorum* subsp. *mucosalis* , however, DNA-DNA hybridization had shown that this species represents a distinct *Campylobacter* species (105). The role of pathogenicity of this species in humans is still unknown.

Camyplobacter rectus cells are plump, straight rods; its cellular surface is covered with a distinctive array of hexagonal, packed, macromolecular subunits. This species is considered as a putative periodontal pathogen. Periodontal diseases in pregnant women may contribute to preterm low-birth weight, however, no conclusive evidence has shown *C. rectus* to be involved (105).

Campylobacter showae cells are multi-flagella straight rods, which consist of polar bundles of two to five flagella. The pathogenicity of this species is unknown even though it has been isolated from human dental plaque, infected root canals, and periodontal lesions (105).

Campylobacter sputorum was originally described as *Vibirio* species but later it was classified as *C. sputorum.* Initially this species had two subspecies, *C. sputorum* subsp. *bubulus* and *C. sputorum* subsp. *sputorum.* However, *C. sputorum* was sub-divided into different infrasubspecifies (biovars), when the two subspecies and *Campylobacter fecalis* were shown to have similar DNA homology. The biovars are classified by the presence of catalase and urease activity. *C. sputorum* bv. sputorum does not produce either catalase nor urease , *C. sputorum* bv. faecalis produces catalase, but not urease, *C. sputorum* bv. paraureolyticus produces urease, but not catalse (105, 113).

Campylobacter upsaliensis is another catalase-negative *campylobacter* besides *C. helveticus*. *C. upsaliensis* is an enteric pathogen in canines, felines, and humans. However, some strains of *C. upsaliensis* have been associated with human abortion, breast abscess, haemolytic-uremic syndrome and prosthetic knee infection (105).

Campylobacter jejuni is the main *Campylobacter* species that causes gastroenteritis in humans, followed by *C. coli. C. jejuni* is the predominant species among other *Campylobacter* species in commercial broiler farming (10, 131). *C. jejuni* has also been found in wide range of hosts such as cattle, pigs and sheep (80, 98, 100, 135) *C. jejuni* is further divided into two subspecies. *C. jejuni* subsp. *doylei* and *C. jejuni* subsp. *jejuni.* The biochemical differences between these two subspecies is the absence of nitrate reduction, cephalothin susceptibility and catalase activity (105) The pathogenic role of *C. jejuni* subsp. *jejuni* is usually associated with gastroenteritis in human, however, the pathogenic role of *C. jejuni* subsp. *doylei* is unknown.

Campylobacter infection plays a role in triggering Guillain-Barré syndrome (GBS), an autoimmune disorder of the peripheral nervous system. This neurological disorder is first described in 1916. The first symptoms of this disorder include weakness in the legs, which could spread to the upper body and along with complete loss of deep tendon reflexes. The *C. jejuni* strains in immunodeficiency patients can be transformed into a number of potential GBS-inducible transformants, which exhibit a high degree of genetic and phenotypic diversity that leads to Guillain-Barré syndrome (158).

2.4 Source of Campylobacter in poultry

The intestinal tract of poultry is a good reservoir of *C. jejuni* as it is commensal in poultry, however, it is pathogenic in ostrich (107). *C. jejuni* has been isolated from the intestinal tract, primarily in the lower gastrointestinal tract, and in excreted faeces. The number of cells in the intestinal tract may number from 10^4 to 10^8cfu/g (17).

C. jejuni is attracted to the mucus-filled crypts of the intestinal tract because of its chemotatic behaviour toward mucin (17). Mucin is a group of glycoproteins found in the secretion of the mucous membranes. Hugdahl *et al.,* (1998) found that *C. jejuni* is chemoattracted to both mucin and L-fucose, which are the main chemoattractants in mucus (75).

The *Campylobacter* infection is directly associated with the ages of the birds (52, 97). The older the birds are, the higher the frequency of infections. Most of the *Campylobacter* infections occur when the birds are more than two weeks old (19, 26). Colonization of the whole flock can happen rapidly when few of the birds are infected, usually within a week (52).

2.5 Role of Campylobacter jejuni in biofilm in the poultry industry

Campylobacter jejuni is an important pathogen in the poultry industry. It causes gastroenteritis in humans. Although it is a highly prevalent food pathogen, *C. jejuni* requires complex conditions to survive outside the host. *C. jejuni* is a microaerophilic microorganism that can only survive well in low oxygen level. *C. jejuni* is described as a thermophilic microorganism, however, temperatures above 55°C can rapidly result in cell death (105). However, *C. jejuni* has been able to survive at 4°C (23, 27).

C. jejuni has been found to form and attach onto biofilm (27, 85, 128, 151). Biofilms are defined as an assemblage of microbial cells that are irreversibly associated with a surface and are enclosed in a matrix of primarily polysaccharide materials (47). Biofilm has been found on non-metallic surfaces such as rubber fingers, plastic curtains, and conveyor belts, as well as in metallic surfaces such as stainless steel in poultry processing plant (6, 95, 134, 153). *C. jejuni* has the ability of form biofilm to various degrees, however, some clinical strains do not seem to form biofilm (85). *C. jejuni* that attaches onto biofilm in the poultry farms and poultry processing plants may be an important source contamination of final products (164).

Biofilms may provide the ideal niche for *C. jejuni* survival in the biofilm, as there are microenvironments in the biofilm that are believed to provide ideal conditions for *C. jejuni* (59). Studies have shown *C. jejuni* can survive in unfavourable conditions such as low level of nutrient and increased oxygenation when integrated with biofilm (105). This may be due to the biofilm providing protection from the general environment. This protection extends to antimicrobial agents including tetra sodium phosphate, quaternary ammonia compounds, peracetic acids, and chlorine base disinfectants (152).

The initial stage for biofilm development of *C. jejuni* is cell attachment by the flagella of microorganism. However, flagella of *C. jejuni* may play another role in biofilm development and maturation rather than, in addition to attachment to a surface (128). Peb4 or also known as CBF2 is a cell-binding protein that is involved in cell adhesion. Peb4 may affect the expression of flagella, motility, chemotaxis, and transport systems, which are required for biofilm formation in *C. jejuni*, thus, deletion of this protein may reduce the biofilm formation (8).

Carbon starvation regulator A (CsrA) is a homodimeric RNA-binding protein in *C. jejuni* that is not only responsible for repression and activation of stationary-phase metabolism but also is a regulator of virulence determinants, including host cell invasion and biofilm formation (53). Deletion of CsrA in *C. jejuni* leads to reduced motility of *C. jejuni*, which in turn reduces the cell attachment.

The *luxS* gene in *C. jejuni* also plays a role in biofilm formation demonstrated by reduced biofilm formation, when the *luxS* gene is deleted. The *luxS* gene encodes the final enzyme in the biosynthetic pathway for AI-2 production, which is thought to be used for interspecies communication (48). This suggests biofilm formation is dependent on AI-2 production (128).

2.6 Transmission of Campylobacter in poultry industry

The main source of *C. jejuni* infections in the human gastrointestinal tract is from the consumption of food contaminated with raw poultry. A broiler is a chicken that is especially bred and raised for meat consumption. Therefore, by eliminating the *Campylobacter* colonization in the broilers, the occurrence of *Campylobacter* infection in humans can be reduced (155). The main route for *Campylobacter* transmission in the broiler farms is by horizontal transmission. Although vertical transmission is possible, it is still debatable at present (9). The horizontal transmission can occur at the poultry farm and the poultry processing plant.

2.6.1 Poultry farm

Poultry flocks can reach 30,000 to 45,000 birds per shed. If there are a few infected birds present in the shed, within a week the whole flock might be colonized as *C. jejuni* can spread rapidly (52). In addition, external sources of contamination may occur in the sheds. These can include the general environment and human activities.

2.6.1.1 General environment

There are several different types of *Campylobacter* strains that found in the environment surrounding the broiler farm and they have similar genotypes as the *Campylobacter* strains isolated from infected humans (26, 54).The *Campylobacter* strains can be introduced into the shed by various carriers such as rodents, birds and others, and also by farm workers (66).

2.6.1.2 Air

C. jejuni has been isolated from the air in the shed as well as the air exiting the shed (19, 26). *C. jejuni* can be spread from one shed to another during cleaning, as *C. jejuni* may be mixed with the dust particles, and travel to a new shed. However, there is assumption that *C. jejuni* cannot survive long in the air due to the dehydrating nature of the air (107). The relative humidity in the sheds is about 70%, and it has been suggested that reduction of the relative humidity may reduced the contamination of *C. jejuni* in the sheds (46, 96).

2.6.1.3 Feed and Litter

Fresh feed and litter does not support the growth of *C. jejuni* because of the dry condition, which is considered to be lethal to the *C. jejuni* (107). However, *C. jejuni* has been isolated from dirty feed and wet litter. Heavily contaminated feeding pans with feathers, litter and faecal materials can be good reservoirs of *C. jejuni.* A dirty feeding pan might be contaminated with *C. jejuni* when in contact with infected birds, as the conditions in the pan are suitable for microbial growth, thus, contaminating the feed (66).

C. jejuni has been detected in wet litter (66). Heavily contaminated wet litter with faecal material and feathers, and the caked litter, especially around the water pans, can also be a good source of *C. jejuni* contamination. Therefore, bad management of the litter can cause *C. jejuni* contamination in the flock (118).

2.6.1.4 Water

C. jejuni has been isolated from drinking water, as *Campylobacter* species can survive in the water (19, 87, 107). Water contamination usually happens after the colonization of the flock. The water system is an easy means of rapid spread of the infection to birds (52). The contamination of the water system can occur when drinking water is in contact with the infected birds. Contamination from the water source may constitute a risk, albeit relatively low, of colonization for broiler flocks, as *C. jejuni* may subject to environmental stress and chlorination in the water, which may injure or kill *C. jejuni* (107).

C. jejuni can degenerate into a form that is viable but non-culturable when is under water-related environmental stress. However, the infectivity of *C. jejuni* in this form is still debatable. There are no conclusive data on the infectivity of this form, even though there are reports on human outbreaks caused by *C. jejuni* in this form. The infectivity of the viable but non-culturable *C. jejuni* may be strain dependent (107).

2.6.1.5 Pests

Cross-contamination with *C. jejuni* between pests and broilers can happen. The environment surrounding the broiler farm is a cocktail of microorganisms due to spilled feedstuff, waste, and effluent. *C. jejuni* can be isolated from the excreted faeces of rodents especially the intestines of mice that are found near the broiler farm (107). However, this poses a low risk of transmission to the environmentally controlled housing system, as the whole shed is covered and is built with pest-proof materials.

2.6.1.6 Animals

Wild and domestic animals around the broiler farm can also be a source of *C. jejuni*. Although these animals are not in direct contact with the poultry, they may excrete *Campylobacter*-infested droppings in the surroundings of the broiler farm, resulting in cross-contamination (107). Cattle that are kept close to the broiler farm may be a source of *Campylobacter* infections, as gentoypically identical strains have been found in cattle that are near the broiler farm (26, 107).

2.6.1.7 Birds

Wild-birds can be a source of *Campylobacter* infection, as many wild-birds such as sparrows, waterfowl, pigeons, and passerines are colonized with *C. jejuni*. The prevalence of colonization may be dependent on age, species, habitat, seasons and migratory behaviour (107). Although the wild-birds do not have access to the shed, they may excrete *Campylobacter*-infested droppings near the shed, as certain strains of *C. jejuni* are similar to those found in infected broilers (66, 107).

2.6.1.8 Insects

Insects such as flies, darkling beetles, and cockroaches are proven carriers of *C. jejuni* (16, 107, 118, 140). The insects might become *Campylobacter* carriers due to cross-contamination with other sources such as faecal droppings, and *C. jejuni* may survive on or within the carriers for few days (66). The frequency of cross-contamination between insects and broilers within the shed is higher than spreading to other sheds by insects, as most of the insects tend to be trapped inside the shed. Flies can also transmit *C. jejuni* when there is a reservoir of *C. jejuni* such as contaminated waste located near the shed (118).

2.6.1.9 Human Activities

Human activity may contribute to the horizontal transmission of *C. jejuni* (52, 66, 107). Farm staff need to move in and out of the sheds because of their work. *C. jejuni* can potentially be carried into the house from the external environment via boots, external clothes and equipment. The boots of farm staff are usually contaminated with *C. jejuni* when the flock has been infected (66, 107).

The first birds in the flock to be colonized are closest to the doors that are used by the farm staff and would be consistent with the trafficking of *C. jejuni* into the shed by farm staff (107). Furthermore, dirty equipment may be contaminated with *C. jejuni*. Good biosecurity and a hygiene barrier are needed in the broiler farms to minimize the risk of cross-contamination. All the equipment that is taken into the sheds needs to be cleaned and disinfected (107).

2.6.2 Processing Plant

The numbers of birds processed in a processing plant per day are in the thousands. Processing plants may play some role in the contamination of the final poultry products. *C. jejuni* contamination can occur while holding and during processing of the live birds in the processing plant (82). High levels of microbial contamination have been isolated from the crates, processing equipment, and the environment of the processing plant (2, 3, 20, 57, 82, 142).

Please refer to Appendix 1 for a simple flow diagram of poultry processing.

2.6.2.1 Transporting and Holdings

Contaminated crates can lead to *C. jejuni* contamination of birds during transport and holding at the processing plants (71, 111). *C. jejuni* have been detected in the organic matter on the crates, even though the crates have been washed (142). The faecal droppings from the infected birds can contaminate the crates as well as the non-infected birds during transport and holding. The crates are usually stacked on top of each other, thus, droppings from infected birds at the top crate may infect the birds in the lower crates.

2.6.2.2 Scalding

Cross-contamination can occur prior to scalding, as *C. jejuni* can be found on the skins and feathers of the live birds. Many microorganisms can enter the water as birds are moved continuously in the scald tank (30). The contamination may be due to contamination from the hands of workers (already contaminated from a previously positive flock) as the birds are placed on the line or possibly from the stun water (142).

2.6.2.3 Defeathering

The defeathering process creates an aqueous aerosol of bacteria and other solids, which may disperse onto other carcasses that both precede and follow it, and contaminate the equipment (2, 3, 5, 57). Worn-out rubber picker fingers could provide favourable growth condition to microorganisms. The rate of isolation of *C. jejuni* decreases during the scalding process (58°C for 70 s). The carcass is re-contaminated at the defeathering process because dirty feathers or faeces which leak from the intestine are mixed in by the rubber picker fingers of the defeathering machine (114).

2.6.2.4 Chilling

The high prevalence of contaminated carcasses observed after the carcasses leave the water chiller indicates that chiller may be a source of cross-contamination, even though this water is considered to be of drinking quality (57). This can be explained by the fact that there is a gradual accumulation of microbial contamination as the low free chlorine content in the chilling tank is insufficient to inactivate microbial contamination.

2.6.2.5 Evisceration

C. jejuni has been isolated from the conveyor belt of the evisceration line. This suggests that evisceration, a process during which rupture of the viscera with extravasations of intestinal content can occur, may be responsible for cross-contamination that would lead to a substantial increase in contamination during processing (57, 114). The intestinal tract of poultry is a good reservoir of *C. jejuni* as it is commensal in poultry (107).

2.6.2.6 Airborne

The air in the processing plant can be a potential vector for microbial transmission (2, 123). The dispersal of loosely attached microbes on the moist surface skin of the carcass to the air is strongly influenced by the mechanical action of the defeathering machine, such as vigorous rotary action of the plucking disc and water (2). The evisceration area is more highly polluted by airborne *C. jejuni* than the hanging area. This could be explained by the use of UV light in the hanging section to control the microbial contamination of the air (123). It appears that *C. jejuni* can be airborne and contaminate the carcasses as well as the workers in the processing plant.

2.6.2.7 Packing

C. jejuni has been isolated from the tables in the packing sector due to the high microbial content once the carcasses have been processed (57). This may result from the re-contamination of the carcasses as they pass through the processing line (114). The hands of the workers may be contaminated with *C. jejuni* when handling contaminated carcasses, thus spreading *C. jejuni* onto other carcasses.

Chapter 3 Introduction to Biofilms

3.1 Introduction

Biofilms can be a problem to the poultry industry, as biofilms have been found on the water systems in the poultry farm and stainless steel in poultry processing plant (6, 7, 37, 77, 134, 141). The biofilms do not only harbour the microbial populations but also act as sources of cross-contamination (164).

Biofilms are an assemblage of microbial cells that are irreversibly associated with a surface and are enclosed in a matrix of primarily polysaccharide materials (47). Biofilms may contain non-cellular materials such as mineral crystal, corrosion materials, and clay or silt particles in the matrix. These non-cellular materials are incorporated into the biofilms from the surrounding environment in which the biofilms are formed. Biofilms can be found in virtually all aquatic ecosystems that can support microbial growth, which include natural aquatic systems, such as streams or rivers, and artificial aquatic systems, such as industrial or potable water system piping (41, 47, 147).

Biofilms can be composed of coccoid microorganisms that are associated with extracellular polymeric substance matrix, which can be found on medical devices. Most biofilms are composed of mixtures of microorganisms which lead to interspecies and intraspecies interactions, and to the general complexity of the macromolecular mixture (147). Biofilms can be formed by monospecies such as *Pseudomonas aerouginosa, Staphylococcus epidermidis, Salmonella Enteritidis, Vibrio cholerae, Streptoccus gordonii,* and *Burkholderia cepacia* (85).

Biofilms can create microenvironments, which may provide a safe haven for survivability of microorganisms. Mature biofilms demonstrate a complex 3-dimensional structure with numerous microenvironments differing with respect to osmolarity, nutritional supply and cell density (59). In addition, biofilms may play a role in protecting the microbial cells during sanitising, as antimicrobial agents may fail to penetrate the biofilm. The exopolysaccharides in biofilms can interact with antimicrobial agents and protect the cells, either by preventing access of the compound or by effectively reducing the concentration, however, the protective effects are probably limited (59, 147).

3.2 Structure of biofilm

Expolysaccharides (EPS) may account for 50% to 90% of the total organic carbon in the extracellular polymeric substance formed by the microorganisms. EPS can be considered as the primary matrix of biofilms (47).

Most of the EPS possess backbone structures that have 1, 3- or 1, 4-β-linked hexose residues. The EPS tends to be rigid in structure, less deformable, and either poorly soluble or insoluble due to the 1,3- or 1,4-β-linked hexose residues (47, 147). These EPS molecules may be present as gel due to the entanglements of long chains of stiff macromolecule and also, in some polymers, to the ionic environments (147). However, there are some EPS that are flexible due to the 1, 2-α- or 1, 6-α-linkages in the polysaccharides. The structures of the polysaccharides determine the primary conformation of the EPS.

The chemical and physical properties of the EPS vary from one microorganism to another. The properties of EPS are also depending on the environment and type of microorganisms that form the biofilms. The EPS of biofilms is not generally uniform, as natural biofilms usually contain a mixture of microorganisms. The amount of EPS produced by the microorganism varies, as each microorganism may synthesize a different composition of EPS and at different rates within the biofilm. Therefore, the physiological state of the biofilm is dependent on the varying proportions of the different polysaccharides and the proportions of different microorganisms within the biofilm.

The production of EPS increases with the aging of biofilms. The production of EPS is affected by the nutrient status of the surrounding medium and slow microbial growth also enhances the EPS production (47). In addition, the thickness of the biofilms can be affected by the amount of EPS produced and by the number of microorganisms within the biofilms. Monocultures of either *Klebsiella pneumoniae* or *P. aeruginosa* biofilms in a laboratory reactor are thinner compared with biofilms containing both of the species (81).

EPS is highly hydrated as it can incorporate a large amount of water into its structure by hydrogen bonding (47). Although some EPS may be hydrophobic due to the compositions of the polysaccharides and the tertiary structure, most of the EPS are both hydrophilic and hydrophobic. The properties of the matrix are different and this leads to the wide differences in properties found in biofilms (147).

Most of these EPS are polyanionic due to the presence of either uronic acid such as D-glucuronic, D-galacturonic and mannuronic acids, or ketal-linked pryruvates in the polysaccharides (47, 147). The polyanionic property is important as it forms a cross-link between the polymer strands and divalent cations such as calcium and magnesium, which provide greater binding force in developed biofilms.

3.3 Biofilm formation

Before biofilm formation can happen, the microorganisms are required to attach to the surface. The solid-liquid interface between a surface (e.g., metal, plastic, glass, gut) and aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms (47). The rate of attachment and the avidity of the interaction reflect an interplay between microbial and substratum surface characteristic, previous conditioning of the substratum and fluid sheer stress (41).

3.3.1 Characteristic of surface

The characteristics of the surface may influence the rate of attachment by the microorganisms. The rate of attachment increases as the surface roughness increases, as this is due to the diminished shear forces, and rougher surfaces (47). The physiochemical properties of the surfaces may also play a role in the attachment. The surfaces of the microorganisms and the materials possess an overall negative charge. In order for attachment to take place, the resulting electrostatic repulsion barrier must be overcome by an attractive force (41).

Microorganisms attach more rapidly to hydrophobic, non-polar surface such as Teflon and other plastic, than to hydrophilic materials such as glass or metals (18, 47). In addition, the surface composition also influences the attachment of the microorganisms because some elements in the material are susceptible to microorganism attachment, such as iron, magnesium, and calcium (6).

Previous conditioning of the substratum can result in chemical modification on the material surface, thus, affecting the rate and extent of attachment. Conditioning happens when the material surface is exposed in natural waters, and adsorb organics that are found in the water (41). Conditioning can also occur in human host, where conditioning can form on tooth enamel surfaces in the oral cavity and is due to the proteinaceous conditioning film called “acquired pellicle” (47).

3.3.2 Surrounding environment

Different microorganisms will form biofilms at different nutrient concentrations. P. *aerouginosa* and *P. fluorescens* will form biofilms under almost any nutrient concentration that allow growth, while some strains of *Escherichia coli* K-12 and *V. cholerae* will not form biofilms in low concentrations of nutrient. However, *E. coli* O157:H7 can form biofilms in low concentrations of nutrient (110).

Seasonal effects may play a role in the biofilm formation in different aqueous systems, as this may be due to the fluctuation of temperature in the aqueous systems (47). Biofilms are also evident in high-temperature environments, such as terrestrial geothermal settings and hydrothermal vents (129). Therefore, mesophilic microorganisms, such as *Pseudomonas species, E. coli* and *S. epidermidis,* and hyperthermophilies, such as *Thermoga maritime*, *Thermococcus litoralis* and *Archaeoglobus fulgidus* can form biofilms in the environment (126).

3.3.3 Flow velocity of environment

The flow velocity of the aqueous medium plays a role in the rate of attachment. The region of flow immediately adjacent to the substratum/liquid interface is termed the hydrodynamic boundary layer, which is dependent on the linear velocity. The region outside the boundary layer is characterized by substantial mixing or turbulence (47). The microorganisms must traverse the boundary layer before they can attach to the surface. As the boundary layer decreases, the velocity increases, and the turbulence outside the boundary layer increases. Therefore, high flow velocity can increase the rate of attachment. However, high velocity can result in detachment of the microorganisms from the surface due to the exertion of substantial shear force on the attaching microorganisms.

3.3.4 Presence of flagella and fimbriae

The presence of fimbriae in the microorganisms influences the rate and extent of attachment of microbial cells. Most fimbriae contain a high proportion of hydrophobic amino acid residue, which contributes to the cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum (47)

In addition, the flagella from a number of microorganisms also play a significant role in the rate of attachment onto the surface (128). Motile microorganisms have higher rate of attachment and biofilm formation than non-motile microorganisms. Flagella apparently play an important role in attachment in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum (47, 110).

3.3.5 Quorum Sensing

Quorum sensing or cell-to-cell signalling has been documented to play a role in cell attachment and detachment from biofilms (47, 128). Xie *et al.,* (2000) demonstrated that certain plaque bacteria, such as *Streptococcus cristatis*, can modulate the expression of the genes encoding fimbrial expression (fimA) in *Porphyromonas gingivalis*, thus, preventing *P. gingivalis* from attaching to biofilms (169).

Davies *et al*., (1998) demonstrated that two different cell-to-cell signalling systems in *P. aerouginosa* were involved in biofilm formation and they are the lasR-lasl and the rhlR-rhll (vsmR-vsml) (40). The cell-to-cell signalling systems *in P aeruginosa* are not involved in the initial attachment and growth stages of biofilm formation, but are involved with biofilm differentiation, when there is a sufficient population density.

Li *et al.,* (2001) demonstrated that induction of genetic competence (enabling the uptake and incorporation of exogenous DNA by transformation) is mediated by quorum sensing in *S. mutans.* Transformational frequencies are 10-600 times higher in biofilms than planktonic cells (94). The living cells are also able to acquire chromosomal DNA from dead cells of the same microorganisms.

3.4 Summary

Microbial growth occurs predominantly as biofilm. The environment within a biofilm community provides constituent microorganisms with many advantages that promote their survival. Any microbial study, should consider the effect of a biofilm community.

Chapter 4 Introduction to specific microorganisms used in this study

4.1 Introduction

Poultry meat is associated with a variety of pathogenic microorganisms. Most of the microorganisms originate from the farm and farm environment. Many common microorganisms in the general environment are also common inhabitants of animals' intestine and their presence may be transient or long term (88). The contamination of poultry carcasses with microorganisms can occur during farming, processing, packaging, distribution, and preparation of food for consumption. Controlling these pathogens requires good hygiene throughout food manufacture.

Salmonella is the predominant serious food-borne pathogen worldwide. Poultry and poultry products are often the vehicle for salmonellosis (133). However, *Campylobacter* is the predominant food-borne pathogen in poultry and poultry products. Besides *Salmonella* and *Campylobacter*, there are other pathogenic microorganisms such as *Escherichia coli*, and *Staphylococcus* spp. that may also be associated with poultry. These pathogens may also be responsible for disease in chickens.

In this study, five microorganisms including pathogens and non-pathogens, were used alongside *Campylobacter jejuni.* These five microorganisms are usually present in the environment and are known to have the ability to form biofilms.

4.2 Escherichia coli

Escherichia coli, an organism of intestinal origin, is associated with food-borne infection in humans and domesticated in animals. *E. coli* is a common inhabitant of the intestinal tract of the chicken (22, 83). *E. coli* predominates the cecum of chicks during the first day after hatching (83). *E. coli* is abundant in chicks but it usually wanes as the birds mature (143). Only certain strains of *E. coli* show virulence factors such as adhesive ability, aerobactin production, serum resistance and presence of ColYplasmid, which can cause diseases (22).

The avian pathogenic *E. coli* (APEC) strains always infect the respiratory tract, with bacteramic strains passing through the mucosa and entering the blood stream, which can cause a variety of disease in poultry (162). The most common form of APEC infections is colibacillosis, which is characterized as an initial respiratory infection such as airsacculitis. Avian colibacillosis initiates in the upper respiratory tract, following the injury of the tracheal epithelium by viruses or mycoplasma, and then colonization of the air sac and lungs by *E. coli* (104). This is usually followed by a generalized infection such as perihepatitis, pericarditis and septicemia(102).

Most of the APEC associated with colibacillosis in domestic poultry belongs to a limited number of serotypes, usually O1, O2, and O78 (73, 102, 162). The strains also exhibit certain patterns of carbohydrate fermentation and antibiotic resistance and express specific adhesions (162). Several potential virulence factors have been associated with APEC such as type 1 (F1A) and P(F11) fimbriae, curli, the aerobactin iron-sequestering system, K1 capsular antigen, temperature-sensitive hemagglutinin (TsH), and resistance to the bactericidal effect of serum (102). A specific region of the chromosome in *E. coil* is likely to be associated with the virulence of APEC strains.

The broiler farm may be a source of *E. coli* contamination. APEC strains are not considered pathogenic to humans. However, poultry is highly susceptible to infection with *E. coli* O157: H7, a highly pathogenic microorganism causing hemorrhagic enteritis in humans (133). Low level of *E. coli* O157: H7 can colonize ceca, and can be excreted in feces (136). There are few factors influencing the prevalence of *E. coli* in the broiler farms such as the crowding of the birds, dirty litter, and poor sanitation (143, 156).

E. coli infections in broilers have been treated with antibiotics. However, they are now ineffective due to the bacterium's resistance (15). Although antibiotic susceptibility may return with the discontinuance of antibiotic use, antibiotic-resistant bacteria can persist long after the removal of the antibiotic.

E. coli is a commonly monitored microorganism in poultry processing plants, it is regarded as an indirect indicator of fecal contamination (63, 133). The levels of *E. coli* associated with poultry carcasses are dependent on the level of fecal contamination in the live birds, length of time and temperature of scalding, efficiency of evisceration, bacterial load and temperature of the immersion chiller water, and hygienic practices in the abattoirs (63). There are varieties of strains isolated from the contaminated carcass during process, due to multiple sources of contamination of the carcass with *E. coli*. Consumption of the contaminated carcasses can cause food poisoning in humans (156).

4.3 Enterococcus faecalis

Enterococcus species belong to the family Enterococcaceae. This genus was originally classified as group D *Streptococcus*, however, in 1984, it was reclassified as *Enterococcus* (92). The change of the classification was required when nucleic acid hybridization studies showed that group D *Streptococcus* was distinctly different from *Streptococcus* (159).

The general characteristics of *Enterococcus* spp. are Gram-positive, non-spore forming ovoid cells, single, or diplococci, and non-motile. Although *Enterococcus* spp. is a facultative anaerobic microorganism, *E. faecalis* is well adapted to aerobic conditions. The biochemical characteristics of *E. faecalis* include the ability to ferment glucose without gas production, catalase negative, reduction of litmus milk and inability to liquefy gelatin.

Enterococcus spp. are usually found in the gastrointestinal tract of mammals, and from the environment. Although *Enterococcus* spp. have complex nutritional requirements, they are resistant to environmental stress. They can survive a variety of temperatures, as many of *Enterococcus* spp. are psychrotrophic and can survive as low as 1°C, and some strains can survive at 50°C (92). *E. faecalis* is usually associated with noscocomial infections such as urinary tract infections, bacteremia, wound infection, and endocarditis.

E. faecalis is resistant to many antibiotics such as penicillin, ampicillin, piperacillin, imipenem and vancomycim. *E. faecalis* acquires antibiotic resistance through the exchange of resistance-coding genes carried on conjugative transposons, pherome-responsive plasmids, and other broad host-range plasmids (76).

Vancomycim resistance *Enterococcus* spp. are usually found in clinical, veterinary and food specimens (78). The increase in vancomycim resistance *Enterococcus* spp. may be due to the increased use of antimicrobial agents in medicine (157). Two of the most common types of vancomycim resistant enterorocci in United States are VanA (resistance to vancomycim and teicoplanin) and VanB (resistance to vancomycim) (76). Vancomycim resistant *Enterococcus* spp. may transfer to humans from a poultry source; however, it is more likely to be due to the transmission of resistance genes than microorganisms (139, 145)

Enterococcus spp. have been found in poultry processing plant equipment and in the poultry farm (61, 62, 157, 160). Although *Enterocccus* spp. do not play a role as food pathogens, they are still important to the poultry industry. This is because ***E. faecalis*** may play a role in pulmonary hypertension syndrome (ascites syndrome) in broilers (148, 149). This metabolic disorder is due to insufficient oxygen availability in the body. Symptoms in infected birds are smaller size than healthy birds, loss of feathers in the abdominal region and a shrunken comb. Besides that, the symptoms of ascites syndrome also includes a reluctance to move, dyspneic, cyanotic, right-side cardiac enlargement and variable liver changes (28). The cardiac enlargement includes dilation of the right atrium, sinus venosus, and vena cava as well as the right ventricle and hypertrophy of both the right ventricle and right muscular atrioventricular valve (28). Measurement of right ventricle to total ventricle (RV/TV) ratio can be used to determine the ascites syndrome status of a bird before gross lesions are apparent (13). An RV/TV ratio of > 0.27 is considered an accurate measure of the onset of ascites. The livers in affected birds vary from congested or mottled to shrunken with a grayish capsule and irregular surface.

Ascites syndrome begins to develop whenever there is insufficient oxygen required for metabolism. In order to cope up with oxygen demand, the blood flow is increased, thus, causing pressure in the lungs and pulmonary arteries (33). Right ventricular hypertrophy can develop due to the persistence of increased pressure; this in turn can cause odema in the lung. As pressure persists, the right heart valve will fail to work properly, therefore it will cause a backpressure in the body. The backpressure can cause leakage of plasma from the liver into the peritoneal cavity. Besides that, the backpressure can result in lack of oxygen to the heart muscle causing hypoxic damage and heart failure.

4.4 Pseudomonas species

Pseudomonas species belong to the family Pseudomonadaceae. This family includes the genera *Azomonas*, Azomonotrichon, Azorhizophilus, *Azotobacter*, Cellvibrio, *Mesophilobacter*, *Rhizobacter*, *Rugamonas*, and *Serpens*. The general characteristics of Pseudomonadaceae are oxidase positive, non-fermentative, and polar flagella. *Pseudomonas* spp. are psychrotropic, aerobic, rod Gram-negative microorganisms that can be found in many natural environments, including sand and water. *Pseudomonas* spp. also can be found in the intestines and mouth of man and animals.

Pseudomonas aeruginosa can cause minor infections in healthy humans such as swimmer's ear (otitis externa) and hot-tub folliculitis. Swimmer's ear is an infection of the ear canal, while hot-tub follicultis is an infection of the hair follicles. *Ps. aeruginosa* is also an opportunistic pathogen, which can cause life-threatening illness in immunocompromised patients. It is usually associated with noscocomial infections such as pneumonia, urinary tract infections, and bacteremia.

Pseudomonas spp. are not regarded as food pathogens but are the predominant food spoilage microorganisms. *Pseudomonas* spp.on poultry meat can cause off-odours, off-flavours and slimy appearance due to proteolytic and lipolytic activities during the microbial growth (64). The most common *pseudomonas* spp. that are associated with poultry meat spoilage are *Pseudomonas fluorescens, Pseudomonas putida,* and *Pseudomonas fragii* and cause economic loss for the poultry industry.

Pseudomonas spp. have been isolated from the poultry processing plant such as the spin chiller (64, 160). The temperature in the spin chiller does not exceed 16°C (160). *Pseudomonas* spp. survive well under low temperature, as they are psychrotropic microorganisms, and are able to grow rapidly at refrigeration temperatures (92). Temperature is the most important environmental factor for the growth of *Pseudomonas* spp. (45). *Pseudomonas* spp. may be introduced to the poultry processing plant during the processing of the birds as well as in water and ice supplies (64).

4.5 Salmonella species

Salmonella species are pathogenic, causing gastroenteritis (salmonellosis) in humans and are one of the leading causes of food-borne diseases in the world (133). The symptoms of salmonellosis are nausea, vomiting, severe diarrhea, fever, abdominal cramps and malaise (133). *Salmonella* enterica serotypes are the major cause of human gastroenteritis. In addition, *Salmonella* spp. can cause typhoid fever, and paratyphoid fever.

Salmonella spp. are Gram-negative, 2-3μm long, mesophilic, non-sporulating rod microorganisms (122) and is classified into over 2,000 serovars (35). The serovars are classified by the somatic (O), flagellar (H), and capsular (Vi) antigens in the microorganism (122). Although the serovars of the *Salmonella* spp. have many similarities, they demonstrate different host specificities, epidemiological characteristics and clinical manifestations (35).

Most strains from warm-blooded animals belong to subspecies I: *Salmonella enterica* subspecies Enterica. The most common types of *Salmonella* spp. found in the poultry industry in New Zealand are *Salmonella* Agona, *Salmonella* Typhimurium phage type 101, *Salmonella* Typhimurium phage type 135, *Salmonella* Infantis, *Salmonella* Brandenburg, and *Salmonella* Typhimurium phage type 160 (91). *Salmonella* spp. colonize the intestinal tract of warm-blooded animals. All the *Salmonella* strains exhibit intestinal colonization except *Salmonella* Pullorum and *Salmonella* Choleraesuis (14). *Salmonella* spp. can penetrate the intestinal tract as far as the liver and spleen, which are the main sites for the multiplication of *salmonella* spp. However, the cecum is identified as an ideal organ for salmonella colonization in acute and chronic diseases in chicks (42).

Salmonella infections can occur in chickens where then can cause enteric lesions. There are two types of *salmonella*-infections, which are pathogenic to the chickens and they are pullorum disease and fowl typhoid. The enteric lesions are usually found in the cecum of the birds. Pullorum disease is caused by *S.* Pullorum, while fowl typhoid fever is caused by *Salmonella* Gallinarum (14, 122). Both of these microorganisms are non-flagellate and they have the same antigenic formula (168). Although *S.* Pullorum and *S*. Gallinarum are genomically similar, they have different infection pathways.

Pullorum disease is an acute systemic disease that is usually prevalent in young chicks, and results in a high mortality rate. However, pullorum disease can also infect the adult birds and can cause weight loss, diarrhea and abnormalities of the reproductive tracts (163). The pullorum disease can be transmitted between generations by vertical transmission (38). Fowl typhoid fever is a chronic disease that usually occurs in adults chickens (168). The spread of fowl typhoid fever is due to the consumption of contaminated food or water, which is different from pullorum disease that is transmitted vertically.

Salmonella spp. can be transmitted vertically and horizontally in broiler farms. *Salmonella* spp. can be passed on between parental flocks and broiler flocks. This is due to *salmonella* colonization of the ovary and contamination of egg formation while in the oviduct (38). The contamination is lessened as the egg progresses through the oviduct. However, the egg can be re-contaminated in the lower oviduct, due to penetration of the eggshell.

Horizontal transmission can happen in the hatcheries, broiler farms and during transporting of the birds. In hatcheries, the eggs can be contaminated due to contaminated equipment. There are some factors that can have an effect on the *Salmonella* penetration such as the egg-shell quality, the numbers of pores in the egg-shell, temperature, humidity and vapor pressure (38).

Salmonella spp. can be isolated from free-range farms as well as commercial broiler farms. Both the farm types have a similar prevalence of *Salmonella* colonization (138). However, there is more drug-resistance in *Salmonella* spp. found in broiler farms. This may due to the use of feed-grade antimicrobials. This could result in failure of treatments to control *salmonella* colonization in broiler farms.

The environment within the broiler shed can be a source of *Salmonella* contamination as *Salmonella* spp. can survive long-term in the shed, despite disinfection (166). Dirty litter or re-used litter in the broiler farm can also be a source of *Salmonella* contamination. However, not the whole litter surface is contaminated with *Salmonella* spp.(69), only certain hot spots of the litter are contaminated, usually near the drinking system and where the litter is wet. Therefore, the litter should be dried, and could be achieved by good ventilation of the shed. Fresh, dry, clean wood shavings, when used as litter, have been shown to inhibit the growth of *Salmonella* spp. (116).

Salmonella contamination can also be caused by pests such as flies and beetles. Chickens have a tendency to peck on pests, and they could be infected by pecking of contaminated pests. *S.* Typhimurium has been isolated from the inside and the outer surface of both larvae and adults beetles (140). However, beetles from the shed might not always be the cause of infection, as they can remain as *salmonella*-free. Therefore, the presence of beetles does not necessarily reflect the *salmonella* –contamination status of the flock.

Flies can transmit *Salmonella* spp. rapidly in the broiler farms, as they are always present in the farm. Adult muscoid flies including the common houseflies can carry *Salmonella* spp. *Salmonella* spp. can survive in flies throughout their 4 week lifespan (166). Flies that are exposed to contaminated material such as manure, feed and water, are capable of transmitting *Salmonella* spp.

Human activities in the shed can also contribute to the contamination of the flocks. Farm staff can indirectly bring *salmonella* spp. from the environment and -contaminate the sheds. The farm staff who always wears the same clothing on the farm, may spread contamination. Catchers may also contribute to the contamination of the flocks when they are catching the birds for processing (142). The hands of the catcher may be contaminated during catching of *salmonella*- positive chicken and handling of contaminated crates.

Contaminated feed can be a source of *Salmonella* contamination, as the chickens can be infected by consuming contamination feed. *Salmonella* spp. can survive and colonize the intestinal tract of the broilers. The contamination of *Salmonella* spp. can be reduced by providing the broilers with *salmonella*-free feed. Although the feed is dry and has low water activity, *Salmonella* spp. have been isolated from the feed. Types of feed used can also be a factor of *Salmonella* contamination. One study showed that feed pellets can increase the incidence of *Salmonella* contamination in the contents of gizzards and ceca of growing broilers (74). Broilers that are feed with pellets diets tend to have higher pH content in the gizzards compare to mash diets. This high pH content is suitable for the growth of *Salmonella* spp., which have optimum growth at pH 6.5 to pH 7.5.

The practice of feed withdrawal is necessary for the broilers before they are slaughtered. This is to allow the evacuation of the intestinal content which will reduce the incidence of carcass fecal contamination during processing (133). This contamination may be due to the leakage of the crop, which can contaminate the carcass. However, removing the feed, results in an increase in the incidence of Salmonella-positive ceca (127). This is because during feed withdrawal there are alterations in the normal microflora in the crop that are favorable to *Salmonella* spp. proliferation.

A reduction of *salmonella* colonization in the crops during feed withdrawal can be done by feeding the broilers with a glucose-based cocktail (72). This glucose-based cocktail can promote the growth of intestinal lactic acid bacteria, which could lower the pH of the crop, as acidity condition is inhibitory to *Salmonella* spp.

Salmonella contamination of poultry usually happens at the processing plants. When there is a *salmonella*-positive chicken in the flock during slaughtering, the *salmonella*-positive chicken can cross-contaminate the *salmonella*-free chicken. In 1995, *Hargis et al.,* stated that crop contamination is the main source of contamination during processing (32). This is because there are low levels of contamination inside the broiler crops. During evisceration, the crops are more likely to rupture, thus, contaminating the broiler meat.

Once the chicken meat in the processing plant has been contaminated, the consumers may be concerned, as the presence of the viable *salmonella* spp. on the finished poultry product can be a potential source for contamination in the kitchen (167). The cross-contamination may occur between raw poultry and cooked food.

4.6 Staphylococcus species

Staphylococcus species are aerobic, non-motile, clustered, Gram-positive cocci microorganisms. There are two types of *Staphylococcus* spp. due to their coagualase reactions. *Staphylococcus aureus* is coagulase-positive and can produce enterotoxins. *S. aureus* is the only known species isolated from poultry to cause food-borne diseases in humans (106). Consumption of these enterotoxins can generally cause a mild gastroenteritis in humans (133). The characteristic of *staphlyoccal*-food poisoning are vomiting, diarrhea and nausea, the symptoms usually appear quickly after ingesting the enterotoxins (44). However, not all *S. aureus* strains present in processed poultry carcass are cause a for public health concern. This is because the animal strains make a very small contribution to human food poisoning (29)

Few species of coagulase-negative *staphylococcus* spp. such as *Staphylococcus sciuri, Staphylococcus saprophyticus* and *Staphylococcus epidermidis*, have been isolated from the poultry farm (86). They have been isolated from the poultry shed and the nares and skin of chickens. The staphylococcal infections in poultry are considered opportunistic, as they can be pathogenic under the appropriate conditions. Although these *Staphylococcus* spp. do not cause food-borne disease in humans, they can cause avian diseases that is difficult to treat due to multiple antibiotic resistance (106).

Staphylococcus infections are also known as Staphylococcosis The main *Staphylococcus* spp. that causes staphylococcosis is *Staphylococcus aureus*. *Staphylococcus* spp. are normal inhabitants of skin and mucous membranes of poultry, and are ubiquituous in environments where poultry is hatched, reared or processed. *S. aureus* can cause disease problems such as staphylococcal dermatitis (70), septicaemia and skeletal infections (130) such as osteomyelitis (101).

S. aureus has been isolated from the joints, tendon sheath and bones of infected chickens (70, 161). The staphylococcal infections usually occur in adult chicken breeders. It is common to isolate *S. aureus* from the yolk sac, liver and skin of broilers. Staphylococcal dermatitis disease is almost similar to *Clostridium perfringens*-associated gangrenous dermatitis observed in poultry (70, 161).

In 1998, McNamee *et a.,* reported that bacterial chondronecrosis of the proximal end of the femur and/or tibiotarsus, associated with mainly *S. aureus*, was identified as the predominant cause of lameness in two commercial broiler chicken flocks (130). The source of contamination in the broiler shed may be from the hatchery, as the predominant strains exist in both the broilers and the hatchery environment. *S. aureus* has also been isolated from the skin of healthy chicks (99).

The pathogenesis of *S. aureus* may be due to the breakdown in natural defense mechanisms through wounds, injections, minor surgical procedures, or may be due to immunodeficiency in chickens. Infection of *S. aure*us during toe trimming can progress to an ascending infection along the tendons, colonization and inflammation of the joints, and spread to adjacent bone by lysis of cartilage (99), which can cause osteomyelitis. Chickens suffering from osteomyelitis have decreased ability to move and suffer from dehydration and later death.

Broiler farms may be a source of *Staphylococcus* contamination, as *S. aureus* has been isolated from the broilers. The broilers that have been contaminated with *S. aureus* is believed to originate from a human source, where the farm staff may have a role in the spread of *S. aureus* in the broiler farms (29, 130).

Staphylococcus contamination of the poultry product may happen at the processing plants. *S. aure*us has been isolated from the incoming birds, equipment and processed carcasses (21). High levels of *Staphylococcus* contamination on poultry meat can lead to rejection of the meat for use in further processing. Defeathering machinery is the main source of cross-contamination in the processing plant (44). This is because *S. aureus* can be isolated from the feathers of the incoming birds as well as feathers within the pluckers.

These five types of microorganisms are most likely to be found in the poultry environment, as they are usually associated with the poultry industry. Therefore, these microorganisms were selected for this study because of their ability to form biofilms, and different biochemical characteristics.

4.7 Aims and Objective

C. jejuni has been found to attach to preformed biofilms in the watering supplies and plumbing systems of animal-husbandry facilities and animal-processing plants, which can enhance the survival of *C. jejuni* in the environment (7, 27, 85, 128, 150).

C. jejuni in monoculture also has the ability to form a biofilm at both 37°C and 30°C. *C. jejuni* in a biofilm remains viable under atmospheric conditions for several weeks, suggesting that once a biofilm is formed this may provide a survival advantage to cells in the ambient environment (59, 85). *C. jejuni* can attach to various materials such as stainless steel, polyethylene and belting in processing plants (7). This could be a problem to the poultry industry, providing a source of contamination for poultry.

Cleaning and sanitizing is known to be difficult when microorganisms have formed a mature biofilm so this may be one reason for the persistence of *C. jejuni* in the poultry industry (7, 59, 147). The microenvironments and the components of the biofilms protect the microorganisms from antimicrobial agents including physical and chemical treatment, thus, increasing the survival of the microorganisms in the environment.

The overall objective of this study was to investigate the biofilm growth of *C. jejuni* in controlled mixed-microbial populations that are usually found in the poultry industry

The specific aims of this study were to:

1. determine the ability of *C. jejuni* strains with different MLST to form biofilms

2. determine the suitable methods of cell enumeration and recovery from biofilm formed in microtitre plate

3. determine the ability of specific microorganisms (*Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Salmonella* Agona and *Staphylococcus simulans*) to form biofilms in the presence and absence of *C. jejuni*

4. investigate the biofilm growth effect of *C. jejuni* in controlled mixed-microbial populations of specific microorganisms

The expected outcome was to improve the understanding of *C. jejuni* colonization in the poultry industry to help develop future methods to minimise *C. jejuni* contamination in the poultry industry and thus reduce the incidence of campylobacteriosis.

Chapter 5 Materials and Methods

5.1 Bacterial strains

Screening of biofilm formation by *Campylobacter jejuni* strains

The *C. jejuni* strains used to screen for the ability to form biofilm were isolated from poultry, water, and human clinical samples from the Manawatu region, New Zealand, by the Hopkirk Research Institute. The trials were performed on four separate occasions with 12 replicates. The strains are summarised in Table 2.

Table 2 *C. jejuni* (21 MLST sequence types) screened for the ability to form biofilm.

ID number	Source	Sequence types
P133A	Poultry	45
P110B	Poultry	474
P215A	Poultry	520
P216C	Poultry	25
P232B	Poultry	48
P332A	Poultry	52
P175A	Poultry	227
P153A	Poultry	1517
P164A	Poultry	190
P139A	Poultry	257
P143A	Poultry	2345
P156A	Poultry	3609
P195A	Poultry	53
P186b	Poultry	21
H29612	Human	61
H22082	Human	474
H76800	Human	45
H118B	Human	48
H130	Human	190
W131A	Water	177
W126A	Water	694

Cell recovery and enumeration of cells from biofilms

The bacterial strains used to examine the cell recovery methods and enumeration of cells from biofilms were isolated from poultry samples from the Manawatu region, New Zealand, by the Hopkirk Research Institute and Institute of Veterinary, and Biomedical Sciences (IVABs), Massey University. The trials were performed on two separate occasions with duplicates. The strains are summarised in Table 3.

Table 3 The two bacterial strains used in the enumeration of cell recovery from biofilm

ID number	Source	Microorganism
En35	Poultry	*Enterococcus faecalis*
S62	Poultry	*Salmonella* Agona

Biofilm formation by *Campylobacter jejuni* in a controlled mixed-microbial population

The bacterial strains used in the study of biofilm formation by *C. jejuni* in a controlled mixed-microbial population were isolated from the Manawatu region, New Zealand, by the Hopkirk Research Institute and Institute of Veterinary, and Biomedical Sciences (IVABs), Massey University. The strains are summarised in Table 4.

Table 4 Six different bacteria strains used in studying the effect on biofilm formation by *C. jejuni* in a controlled mix-microbial population study

ID number	Source	Microorganism
P110B	Poultry	*Campylobacter jejuni*
En35	Poultry	*Enterococcus faecalis*
EC	Poultry	*Escherichia coli*
Ps13	Water bath	*Pseudomonas aerouginosa*
S62	Poultry	*Salmonella* Agona
ST	Poultry	*Staphylococcus simulans*

The five different microorganisms were mixed into controlled mixed-microbial populations. In total, there were 31 different experiments including 26 mixed species combinations and five individual microorganisms. The trials were performed on two separate occasions with eight replicates. Two of the replicates were used for cell recovery and enumeration of *C. jejuni*. The different combinations are summarised in Table 5.

Table 5 Combinations of microorganisms used in studying biofilm formation by *C. jejuni* in a controlled mixed-microbial population study

Id number	Individual/Combinations of microorganisms
s1	*Ps. aerouginosa*
s2	*E. coli*
s3	*E. faecalis*
s4	*S.* Agona
s5	*S. simulans*
s6	*Ps. aerouginosa*, *E. coli*, *E. faecalis*, *S.* Agona
s7	*Ps. aerouginosa*, *E. coli*, *E. faecalis*, *S. simulans*
s8	*Ps. aerouginosa*, *E. coli*, *E. faecalis*, *S.* Agona, *S. simulans*
s9	*Ps. aerouginosa*, *E. coli*, *S.* Agona
s10	*Ps. aerouginosa*, *E. coli*, *S.* Agona, *S. simulans*
s11	*Ps. aerouginosa*, *E. coli*, *S. simulans*
s12	*Ps. aerouginosa*, *E. faecalis*, *S.* Agona
s13	*Ps. aerouginosa*, *E. faecalis*, *S. simulans*
s14	*Ps. aerouginosa*, *E. faecalis*, *S.* Agona, *S. simulans*
s15	*Ps. aerouginosa*, *S.* Agona, *S. simulans*
s16	*E. coli*, *E. faecalis*
s17	*E. coli*, *S.* Agona
s18	*E. coli*, *S. simulans*
s19	*E. coli*, *E. faecalis*, *S.* Agona
s20	*E. coli*, *E. faecalis*, *S. simulans*
s21	*Ps. aerouginosa*, *E. coli*
s22	*Ps. aerouginosa*, *E. faecalis*
s23	*Ps. aerouginosa*, *S.* Agona
s24	*Ps. aerouginosa*, *S. simulans*
s25	*Ps. aerouginosa*, *E. coli*, *E. faecalis*
s26	*E. coli* ,*E. faecalis*, *S.* Agona, *S. simulans*
s27	*E. coli* ,*S.* Agona, *S. simulans*
s28	*E. faecalis*, *S.* Agona
s29	*E. faecalis*, *S. simulans*
s30	*E. faecalis*, *S.* Agona, *S. simulans*
s31	*S.* Agona, *S. simulans*

5.2 Methods

Most of the methods used in this study were performed using a slight modification of a microtitre plate assay (68). This method is quantifiable, where the optical density of a stained biofilm is measured.

5.2.1 Culture preparation

***Campylobacter jejuni* strains preparation**

C. jejuni strains from the culture collection were streaked onto blood agar plates (Fort Richard) and incubated at 42°C for 48 h in a microaerophilic workstation (atmosphere: N2, 85%, O2, 5% CO2, 10%, MACS VA500 Microaerophilic workstation, Don Whitley Scientific). The aim was to obtain fresh, pure cultures from the *C. jejuni* strains.

After incubation, single colonies were picked and streaked onto fresh blood agar plates. The plates were incubated at 42°C for 48 h in a microaerophilic workstation This was to get good growth on the blood agar.

A loopful of the fresh culture was taken from the blood agar plate and transferred into 10 mL of Mueller-Hinton broth (Fort Richard). The inoculated broths were incubated overnight at 42°C in the microaerophilic atmosphere to build up the inoculum in the broth.

Other bacterial strains

Bacterial strains from the culture collection were streaked onto blood agar plates and incubated at 37°C for 24 h. The aim was to obtain fresh, pure cultures from the bacterial strains. After incubation, single colonies were picked and streaked onto fresh blood agar plates. The plates were incubated at 37°C for 24 h. This was to get a good growth on the blood agar. A loopful of the fresh culture was taken from the blood agar plate and transferred into 10 mL of Mueller-Hinton broth. The inoculated broths were incubated overnight at 37°C to build up the inoculum in the broth.

Enumeration of initial inocula

The overnight cultures were serially diluted (10^{-1} – 10^{-6}). 0.1 mL of the 10^{-6} dilution was plated in duplicate on blood agar plates using an automatic spiral plater (Don Whitley Scientific). The plates for *C. jejuni* were incubated at 42°C for 48 h in the microaerophilic workstation, while other bacterial strains were incubated at 37°C for 24 h. The colonies on the blood agar were counted with a colony counter (Colony counter ācolyte, Symbiosis). The average number of colonies on the blood agar plates were calculated and recorded.

5.2.2 Biofilm formation

Screening strains of *Campylobacter jejuni* for the ability to form biofilm

For the screening of biofilm formation by *C. jejuni* strains, one mL of overnight culture was transferred into 5 mL of Mueller-Hinton broth to provide an inoculum. The wells of 96-well polystyrene plates (Falcon 353072 BD Bioscience) were inoculated with 0.2 mL of this culture and each plate included 12 control wells, which comprised 0.2 mL of uninoculated Mueller-Hinton Broth.

Cell recovery and enumeration of cells from biofilms

For the cell recovery and enumeration of cells from biofilms, one mL of overnight culture was transferred into 5 mL of Mueller-Hinton broth to provide an inoculum. The wells of 96-well polystyrene plates were inoculated with 0.2 mL of this culture and each plate included eight control wells, which comprised 0.2 mL of uninoculated Mueller-Hinton Broth.

Biofilm formation by *Campylobacter jejuni* in a controlled mixed-microbial population

In order to study the effect of different bacterial strains on the formation of biofilm by *C. jejuni*, controlled mixed-cultures were made by transferring one mL of the overnight pure broth culture into a sterile 10 mL bottle. The mixed-cultures were labeled as "without *Campylobacter.*"

Another set of controlled mixed-cultures was made by transferring one mL of the overnight pure culture broth into sterile 10 mL bottle. One mL of the overnight *C. jejuni* culture was transferred into each of the combinations. The mixed-cultures with *C. jejuni* were labeled as *'with Campylobacter."*

One mL of each of the mixed-cultures was transferred into a 5 mL of MHB to provide an inoculum. Eight wells of the 96-well polystyrene plates were inoculated with 0.200 mL of the mixed-culture. The plate also included eight control wells, which comprised 0.200 mL of uninoculated MHB.

Biofilm growth

In order to allow the growth of biofilms, the microtitre plates were covered with a lid and placed inside sealable plastic boxes with microaerophilic pouches (N_2, 85%, O_2, 5% CO_2, 10%, Ngaio Diagnostic). The boxes were placed on top of a bench shaker (Global Science) and incubated with gentle swirling at 30 rpm at 37°C for 72 h. New microaerophilic pouches were put inside the boxes after 48 h of incubation. This was to maintain the microaerophilic conditions inside the box as recommended by the manufacturer.

Biofilm readings

In order to measure the amount of biofilm forming on the plates after incubation, the plates were washed three times with sterile distilled water, and dried at 42°C for 30 minutes. After drying, the biofilm in the wells was stained with 0.200 mL of 0.5% of crystal violet and left on the bench for 15 minutes. The crystal violet was removed by inverting the plates, and the wells were washed three times with sterile distilled water and dried for another 30 minutes at 42°C. The stain was released from the biofilm in the wells by adding 0.200 ml of 98% ethanol. The concentration of the crystal violet in the ethanol was used to determine the relative amount of biofilm by measuring the optical density at 595 nm using a microtitre plate reader (ELx808 Ultra Microplate Reader, Bio-tek instruments, Inc.)

5.2.3 Cell recovery

To recover cells from the biofilms forming in the wells of the microtitre plates after incubation, the biofilms in the microtitre wells were washed with sterile distilled water and replaced with 0.2 mL of Mueller-Hinton broth. The microtitre wells were subjected to different methods of cell recovery (please refer to the following section).

5.2.3.1 Swabbing

To recover biofilm from the wells of a microtitre plate using a cotton wool swab, a sterile swab was pressed against the surface of the well and rotated around the well five times clockwise and another five times anticlockwise. The swab was then placed inside a fresh 10 mL of Mueller-Hinton broth and manually shaken for 30 s.

5.2.3.2 Scraping

To recover biofilm cells from the wells of microtitre plates by scraping, a blade that has been flamed sterilized with 95% ethanol was used to scrape the well surface. After scraping, the microtitre plates were manually shaken for 30 s.

5.2.3.3 Vortex mixing

To recover cells from the wells of microtitre plates by vortex mixing, one sterile glass bead (5 mm^2) was placed inside each well of the microtitre plate and mixed on a vortex mixer for 30 s.

5.2.3.4 Sonication

To recover biofilm cells from the wells of a microtitre plate using sonication, the microtitre plate was placed in a sonicator water bath for 30 s, 60 s, 90 s and 120 s at maximum power and 80% power. After sonication, the microtitre plate was manually shaken for 30 s.

5.2.4 Enumeration of detached cells

After the detaching the cells from the biofilm into the Mueller-Hinton broth, the suspension of cells was serially diluted (10^{-1} – 10^{-6}). 0.1 mL of each dilution was plated on a blood agar plate using an automatic spiral plater. This was done in duplicate and the plates were incubated at 37°C for 24 h. After incubation, the colonies were counted.

For *C. jejuni* enumeration, the cultures were plated on mCCDA agar instead of blood agar plates using an automatic spiral plater. This was done in duplicate and the plates were incubated at 42°C for 48 h. After incubation, typical colonies of the organisms of interest were counted.

5.2.5 Biofilm formation index

The biofilm formation index was used to express biofilm formation by *C. jejuni* in a controlled mix-microbial population study. This index was used because it measures both the biofilm and the cell growth in the microtitre plate (108, 115). Different microorganisms have different morphology and growth rate. By converting the two parameters into an index, comparison of the combinations can be analysed. The biofilm formation index was determined by applying the formula:-

BFI = (AB – CW)/G in which BFI is the Biofilm Formation Index, AB is the optical density of the stained attached microorganisms, CW is the optical density of the stained control wells containing bacteria-free medium only and G is the optical density of cells growth in suspended culture. The following guide was used to interpret the BFI readings (Table6).

Table 6 Semi quantitative classification of biofilm production (P.Naves et al., 2008)

Strong (s)	Moderate (M)	Weak (W)	None(N)
≥1.10	0.70-1.09	0.35-0.69	<0.35

5.2.6 Statistical Analysis

Data were analyzed with SAS software, using SAS analysis of variance (Proc ANOVA) with student-t test or Tukey's test at P = 0.05. The conversions of BFI were performed using the Microsoft Excel software.

5.3 Limitations

The microtitre plate method is an indirect indication of the quantity of biofilm produced, based on the quantity of crystal violet absorbed by the film (25, 43, 117). This assay does not differentiate between living and dead cells. There was some disparity between experiments and it was thought that variation between different lots of plates might be important. The manufacturer's lot numbers of the polystyrene plates were therefore monitored. In addition, the film needs to be thoroughly dry before staining is undertaken and it is important to adhere to the specified volumes and timing of the staining and de-staining steps to ensure consistency.

Cell enumeration by the swabbing method may be compromised as the accuracy of the results depends on the consistency of the researcher (60). In addition, some bacteria may not be counted if injured or viable-but-non-culturable (VBNC) forms (31).

5.4 Advantages

The microtitre plate assay allows rapid analysis of the adhesion and biofilm growth of multiple bacterial strains or the effects of different growth conditions with each experiment and is suitable for fastidious microorganisms such as *C. jejuni* (43). The method is simple, timesaving, and easy to use compared with the Syto9 assay, the fluorescein diacetate (FDA) assay, the resazurin assay, the XTT assay and the dimethyl methylene blue (DMMB) assay (117).

The biofilm formation index can be defined as an estimate of the density of the biofilm that would be generated by a culture of a certain density, as it considers both the bacterial planktonic growth and biofilm accumulation in a single parameter (108, 115).

Although cell enumeration may be compromised by the swabbing method, as only a proportion of cells (normally 10%) are recovered by swabbing, it was timesaving and easy to use (60). This method was suitable for recovering *Campylobacter* spp. from the biofilm in the microtitre plate. This is because the *C. jejuni* is a fastidious microorganism and limited exposure to air is important to ensure maximum recovery of this organism.

Chapter 6 Results and discussion

6.1 Biofilm formation by Campylobacter jejuni

A total of 21 strains of *C. jejuni* strains from poultry, clinical and human sources in the Manawatu, New Zealand, were screened for their ability to form biofilms.

6.1.1 Initial inoculums

All inocula were prepared using the same procedures and the estimated concentration of cells used in the biofilm screening assay was standardised to approximately 10^8 c.f.u/mL by direct cell count.

6.1.2 Biofilm formation by *Campylobacter jejuni*

The ability of *C. jejuni* strains to form biofilm was determined using the microtitre plate biofilm assay (Fig 2). Most of the strains produced some biofilm and many of the strains appeared similar in their ability to produce biofilm (Fig 2). However, some strains MLST-474 (H), MLST-474 (P), MLST-53, and MLST-520 produced particularly strong biofilms. MLST-45 gave inconsistent results in the study and therefore, is not included (Fig 2).

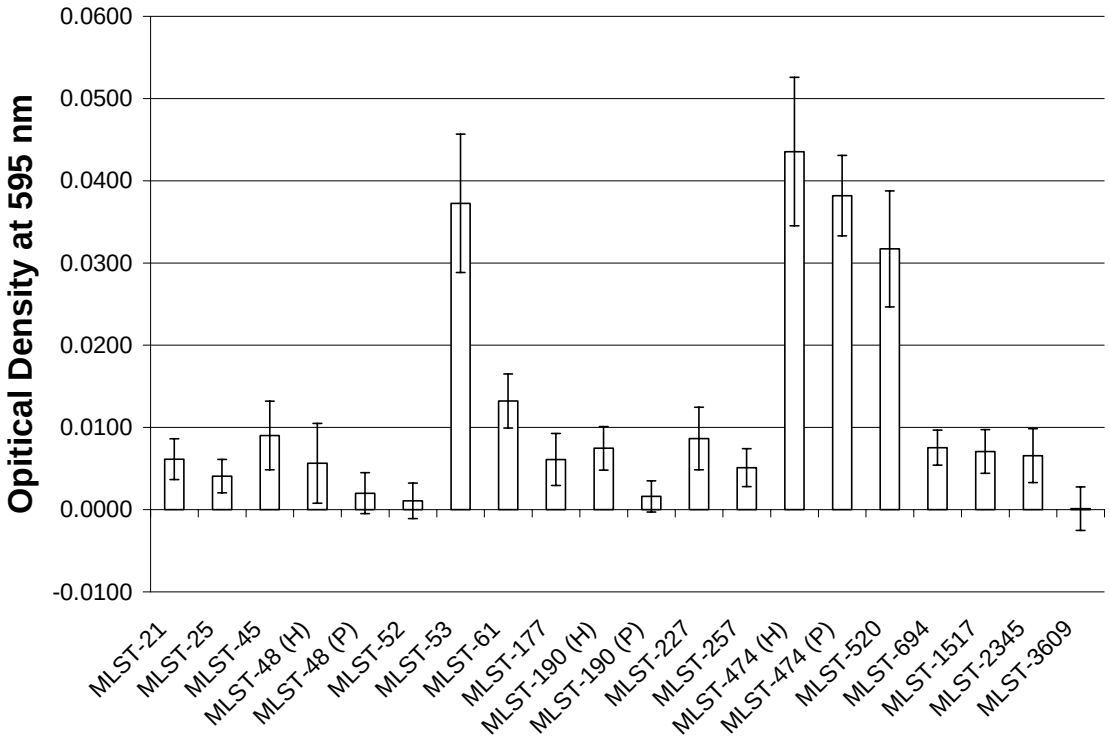

Figure 2 Biofilm formation on polystyrene microtitre plates by *C. jejuni* strains with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. The letter from the MLST represents the source of the strains, (H) is from clinical human, and (P) is from poultry. Trials were performed on four separate occasions, and error bars represent one standard deviation from the mean.

Table 7 Biofilm formation on polystyrene microtitre plates by *C. jejuni* strains with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. Trials were performed on four separate occasions. Means of the trials were calculated.

Source	Multi Locus Sequence Type (MLST)	Mean Optical Density
Human	474	0.0435^{a}
Poultry	474	0.0382^{b}
Poultry	53	0.0373^{b}
Poultry	520	0.0317^{c}
Human	61	0.0134^{d}
Poultry	45	0.0090^{e}
Poultry	227	0.0086^{e}
Water	694	0.0075^{ef}
Poultry	21	0.0075^{efg}
Human	190	0.0075^{efg}
Poultry	1517	0.0071^{efg}
Poultry	2345	0.0066^{efg}
Water	177	0.0061^{efg}
Human	48	0.0056^{efg}
Poultry	257	0.0051^{fgh}
Poultry	25	0.0041^{ghi}
Poultry	48	0.0020^{hij}
Poultry	190	0.0016^{ij}
Poultry	52	0.0011^{ij}
Poultry	3609	0.0001^{j}

Means with the same letter are not significantly different at $P < 0.05$ (T-test).

Most of the strains used in this screening had the ability to form biofilm, with strong biofilm formation observed in human strains (MLST-474 and MLST-61) and poultry strains (MLST-474, MLST -53, and MLST-520), while poultry strains (MLST-3609) did not form biofilm under the conditions of the experiment (Figure 2). Good biofilm formation was observed in MLST-474 from both the human strain and poultry strain; however, they appeared to be different (Table 7). The ability of *C. jejuni* to form biofilm on an abiotic surface may help explain its ability to survive in the environment and act as a source of contamination in the poultry industry. *C. jejuni* may also attach to preformed biofilm, thus increasing survivability in the environment (6, 27, 67, 85, 128, 151). However, the quantities of biofilm produced by *C. jejuni* strains in this screening were much lower compared with other microorganisms in previous studies (34, 68), possibly as a consequence of the type of microtitre plates used in this assay. The plates chosen for this assay were tissue culture plates treated to provide hydrophilic surfaces to clearly differentiate between the strong and weak biofilm producing cells.

6.2 Methods of cell recovery and enumeration cells from biofilms

To examine the cell recovery methods for the enumeration of cells from biofilms, two bacteria, *Enterococcus faecalis* and *Salmonella* Agona were selected from poultry strains as reference strains for all bacteria to be used in this study. These two bacterial strains were used because of their ability to form biofilms and their ability to survive in aerobic conditions (25, 49, 84, 134, 144). *C. jejuni* was not used in this study because of their inability to survive well in the aerobic conditions. The cells recovered from the biofilm were converted to base-10 logarithm values and subjected to ANOVA analysis at $P < 0.05$.

6.2.1 Initial inoculums

The mean concentrations of *E. faecalis* and *S.* Agona cells used as inocula in the cell recovery from biofilm trials were 9.10×10^8 c.f.u/mL and 1.12×10^9 c.f.u/mL respectively.

6.2.2 Comparison of cell recoveries

The mean cell counts for organisms recovered from biofilms in the microtitre plate assay are compared in Table 8.

Table 8 The average number of cells recovered from the biofilm with different methods of removal.

	E. faecalis (log_{10} c.f.u/ mL)	*S.* Agona (log_{10} c.f.u/ mL)	*E. faecalis* and *S.* Agona (log_{10} c.f.u/ mL)
Swabbing	5.2041^{de}	5.6009^{bc}	5.4025^{c}
Scraping	6.1693^{ab}	5.8535^{ab}	6.0114^{ab}
Vortexing	5.4978^{dc}	5.3767^{c}	5.4373^{c}
Sonication at maximum power			
30s	5.8793^{bc}	6.0305^{a}	5.9549^{b}
60s	6.6978^{a}	5.9211^{ab}	6.3095^{ab}
90s	6.6195^{a}	6.2135^{a}	6.4165^{a}
120s	6.3226^{ab}	6.0561^{a}	6.1893^{ab}
Sonication at 80% power			
30s	4.8781^{de}	4.4073^{de}	6.1893^{de}
60s	4.6961^{e}	4.0807^{e}	4.3884^{e}
90s	5.0394^{de}	4.3776^{de}	4.7085^{de}
120s	5.2219^{de}	4.6292^{d}	4.9255^{d}

Means with the same letters in the same column are not significantly different at $P < 0.05$ (T-test).

E. faecalis and *S.* Agona at 6.978l $\log_{10}$ c.f.u/ mL (60 s) and 6.2135 $\log_{10}$ c.f.u/ mL (90 s) respectively (Table 8), were recovered from 72 h biofilms using sonication at maximum power. The counts were lower when the power of sonication was reduced to 80%, with *E. faecalis* at 4.6961 $\log_{10}$ c.f.u/ mL and *S.* Agona at 4.0807 $\log_{10}$ c.f.u/ mL. This may be due to the lower force exerted on the cells in the low power experiments. The greater the power of the sonication, the greater the force exerted on the cells, thus forcing the cells out of the biofilm. However, the liquid in the wells was seen to splash out when full power was used. This could give a false reading, with cells lost from the wells and cross-contamination between wells.

The cell recovery of *E. faecalis* from biofilms with sonication set at maximum power varied with the time of sonication. A low cell count 5.8793 $\log_{10}$ c.f.u/ mL was observed after 30 s exposure with a higher cell count, 6.6978 $\log_{10}$ c.f.u/ mL, observed after 60 s (Table 8). There was no significant difference among the time intervals, 60 s, 90 s, and 120 s with sonication at maximum power or reduced power ($P<0.05$).

There was no significant difference in the cell recovery of *S.* Agona at different time intervals with sonication at maximum power ($P<0.05$). It appears that the maximum power may have removed all the cells, even at the minimum exposure time (30 s). However, there was a significant difference in the cell recovery at different time intervals at lower power where the cell counts after 60 s and 120 s exposures were 4.0807 $\log_{10}$ c.f.u/ mL and 4.6292 $\log_{10}$ c.f.u/ mL respectively (Table 8). The conclusion from these experiments is that the effect of power on cell removal was greater than the time of exposure. In order to use sonication effectively, power and time may need to be optimized to ensure maximum cell recovery with minimal splashing from the microtitre plate wells.

Both *E. faecalis* and *S.* Agona were able to be recovered from biofilm by scraping. Scraping produced the second highest recovery with cell counts of 6.0114 $\log_{10}$ c.f.u/ mL (Table 8). Interestingly, there was no significant difference ($P < 0.05$) between scraping and sonication at maximum power, suggesting that both methods were removing the maximum number of cells from the biofilm.

The vortexing and swabbing methods both gave lower counts compared with scraping and sonication at maximum power. There was no difference between the vortexing and swabbing methods with 5.4373 $\log_{10}$ c.f.u/ mL and 5.4025 $\log_{10}$ c.f.u/ mL cells recovered respectively (Table 8).

Swabbing was chosen as the method to recover *Campylobacter jejuni* from biofilms in used in this study because it was fast and simple compared with the other methods. Even though the numbers of cells recovered from biofilms using swabbing were not as great as scraping or sonication, differences in the number cells recovered was small and the reproducibility of recovery using swabbing was consistent.

6.3 Biofilm formation by Campylobacter jejuni in a controlled mixed-microbial populations

Biofilms have been found in the water systems of poultry farms and stainless steel in poultry processing plant. This may lead to contamination of poultry products with both pathogenic and non-pathogenic microorganisms. In this study, five types of microorganisms associated with poultry and biofilm formation, were used to determine the effect on biofilm formation by *C. jejuni.* The five different microorganisms were mixed into several combinations. These microorganisms were isolated from the Manawatu region, New Zealand.

6.3.1 Initial inoculums

The mean concentration of bacterial cells used as inocula in each trial is given in Table 9. The initial inoculums were standardised to approximately 10^8 c.f.u/mL by direct cell count.

Table 9 The mean initial inoculum used (2 Replicates)

Microorganisms	Average (c.f.u/mL)
Pseudomonas aeruginosa	2.59×10^8
Escherichia coli	2.38×10^8
Enterococcus faecalis	3.04×10^8
Salmonella Agona	1.91×10^8
Staphylococcus simulans	1.69×10^8
Campylobacter jejuni	1.13×10^8

6.3.2 Overall comparison

Comparisons in the total amount of biofilm produced by the different combinations of mixed bacterial species are given in Figure 3.

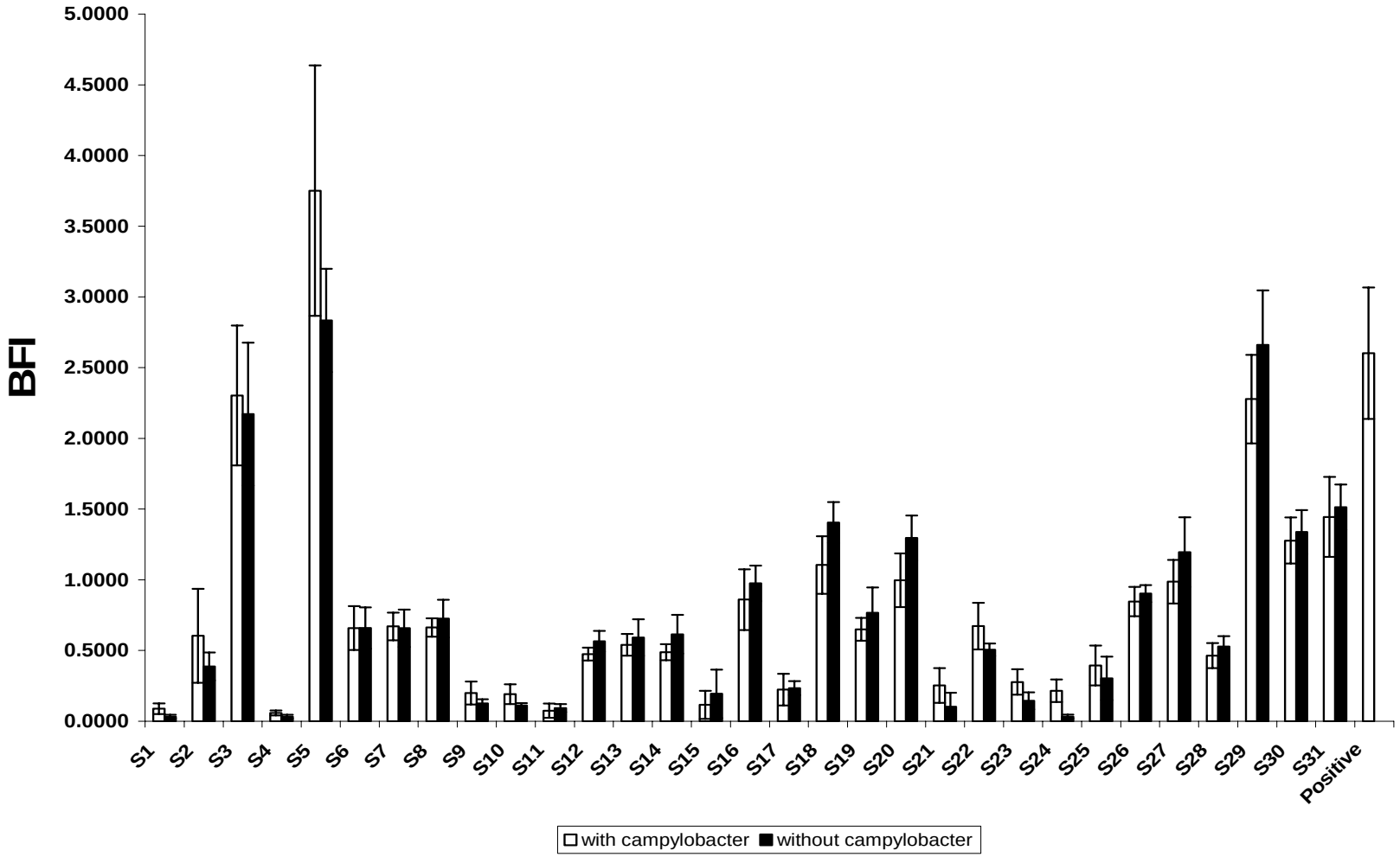

Figure 3 Biofilm formation on polystyrene microtitre plates by different combination with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 31 combinations with *Campylobacter jejuni*. A pure culture of *C. jejuni* was used as positive control. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 10 Biofilm formations by controlled mix-microbial populations. Experiments were performed on two separate occasions. Means of the trials were calculated.

Experiment	Means of BFI		Semi quantitative classification of biofilm production	
	With *Campylobacter jejuni*	Without *Campylobacter jejuni*	With *Campylobacter jejuni*	Without *Campylobacter jejuni*
S1	0.0887	0.0317	None	None
S2	0.6044	0.3873	Weak	Weak
S3	2.3029[a]	2.1715[a]	Strong	Strong
S4	0.0584	0.0318	None	None
S5	3.7515	2.8335	Strong	Strong
S6	0.6585[a]	0.6590[a]	Weak	Weak
S7	0.6703[a]	0.6574[a]	Weak	Weak
S8	0.6626[a]	0.7254[a]	Weak	Moderate
S9	0.1988	0.1270	None	None
S10	0.1912	0.1121	None	None
S11	0.0745[a]	0.0926[a]	None	None
S12	0.4749	0.5655	Weak	Weak
S13	0.5400[a]	0.5919[a]	Weak	Weak
S14	0.4874	0.6146	Weak	Weak
S15	0.1162[a]	0.1940[a]	None	None
S16	0.8596[a]	0.9748[a]	Moderate	Moderate
S17	0.2235[a]	0.2336[a]	None	None
S18	1.1046	1.4047	Strong	Strong
S19	0.6492	0.7669	Weak	Moderate
S20	0.9965	1.2956	Moderate	Strong
S21	0.2535	0.1014	None	None
S22	0.6723	0.5054	Weak	Weak
S23	0.2778	0.1440	None	None
S24	0.2157	0.0325	None	None
S25	0.3944[a]	0.3025[a]	Weak	None
S26	0.8457[a]	0.9030[a]	Moderate	Moderate
S27	0.9861	1.1958	Moderate	Strong
S28	0.4640a	0.5289a	Weak	Weak
S29	2.2777	2.6608	Strong	Strong
S30	1.2774[a]	1.3379[a]	Strong	Strong
S31	1.4444[a]	1.5138[a]	Strong	Strong

Means with the same letters in the same row are not significantly different at $P < 0.05$ (T-test).

There were five different bacteria used in this study besides *Campylobacter jejuni*. The five microorganisms used in this study were *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Salmonella* Agona, and *Staphylococcus simulans*. In total, there were thirty-one combinations with *C. jejuni* in each combination, and each microorganism was present in sixteen combinations. Six out of the thirty-one combinations with *C. jejuni* showed strong biofilm formation, four out of the thirty-one combinations showed moderate biofilm formation, eleven out of the thirty-one combinations showed weak biofilm formation and ten out of thirty-one combinations did not form biofilm.

In this study, strong biofilm formation was observed for the S5 (*S. simulans* and *C. jejuni*) combination with a BFI of 3.75. *S. simulans* was a good biofilm producer, forming a good biofilm on its own. The BFI of *S. simulans* was significantly increased with the addition of *C. jejuni* (Table 10). The S29 (*S. simulans* and *E. faecalis*) combination without *C. jejuni* had the second highest BFI with 2.66, however, with the addition of *C. jejuni* in the combination, the BFI was significantly reduced (Table 10) although it was still a strong biofilm producer.

Ps. aeruginosa and *S.* Agona did not appear to have the ability to form biofilm on their own, as both of them had the lowest BFI compared with the *E. coli, E. faecalis* and *S. simulans*. *Ps. aeruginosa* had the lowest BFI, which might due to its nature as an aerobic microorganism in this microaerophilic environment. Stepanovic *et.al*., (2003) stated that the quantity of biofilm produced by *Salmonella* spp. under microaerophilic conditions was statistically higher than biofilm formed under aerobic conditions, however, the BFI of *S.* Agona in this study was lower compared with the other microorganisms (144). This may due to the dynamic effect of agitation. Agitation may make the attachment of *S.* Agona and subsequent biofilm formation difficult, even though the agitation was set as low as 30 rpm. Note that not all the *Salmonella spp.* may have the ability to form biofilms.

Overall, this study showed that the presence of a variety of other bacterial species found in the poultry environments was generally unaffected by *C. jejuni* in the production of biofilms.

6.3.3 Combination with and without *Pseudomonas aeruginosa*

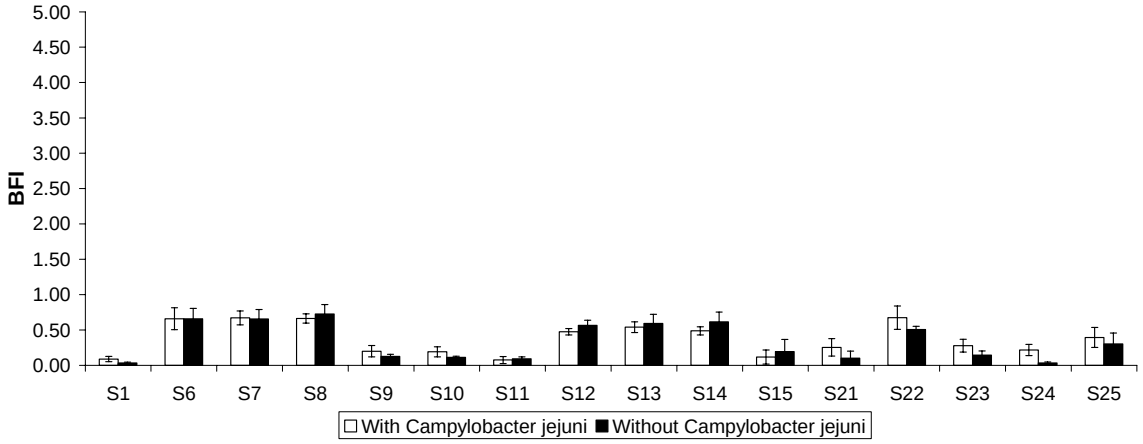

Figure 4 Biofilm formation on polystyrene microtitre plates by different combinations with *Pseudomonas aeruginosa* with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 11 Biofilm formation index (BFI) of combinations with *Pseudomonas aeruginosa*

	With *Campylobacter jejuni* (BFI)	Without *Campylobacter jejuni* (BFI)
S1	0.0887^{jk}	0.0317^{k}
S6	0.6585^{abc}	0.6590^{abc}
S7	0.6703^{ab}	0.6574^{abc}
S8	0.6626^{ab}	0.7254^{a}
S9	0.1988^{ghij}	0.1270^{hijk}
S10	0.1912^{ghij}	0.1121^{jik}
S11	0.0745^{jk}	0.0926^{jk}
S12	0.4749^{de}	0.5655^{bcd}
S13	0.5400^{bcde}	0.5919^{abcd}
S14	0.4874^{de}	0.6146^{abcd}
S15	0.1162^{ijk}	0.1940^{ghij}
S21	0.2535^{fhgi}	0.1014^{ijk}
S22	0.6723^{ab}	0.5054^{cde}
S23	0.2778^{fgh}	0.1440^{hijk}
S24	0.2157^{ghij}	0.0325^{k}
S25	0.3944^{ef}	0.3026^{fg}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

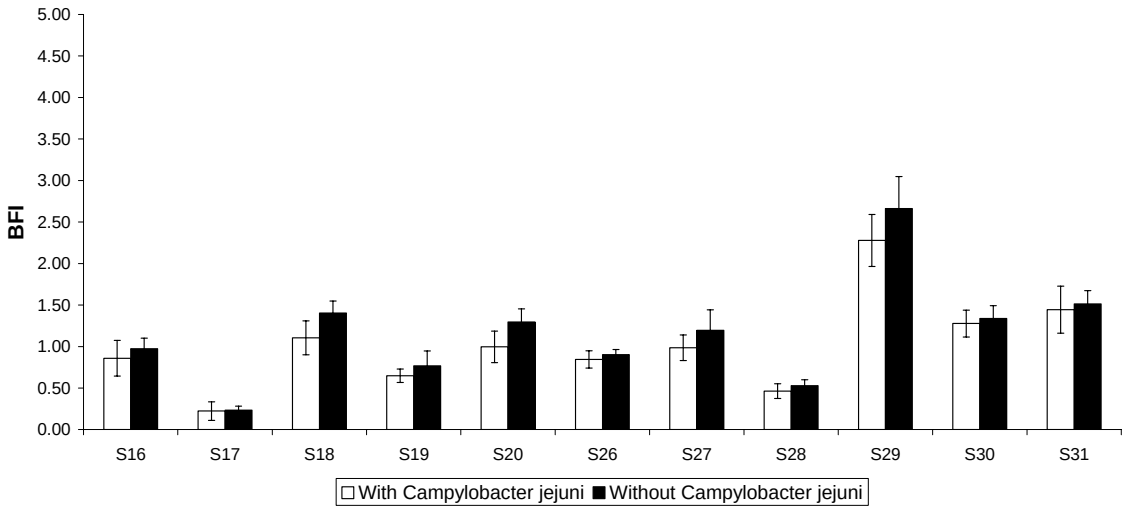

Figure 5 Biofilm formation on polystyrene microtitre plates by different combinations without Pseudomonas aeruginosa with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 12 Biofilm formation index (BFI) of combinations without *Pseudomonas aeruginosa*

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S16	0.8595^{ghi}	0.9748^{fgh}
S17	0.2235^{l}	0.2336^{l}
S18	1.1046^{efg}	1.4047^{cd}
S19	0.6492^{ijk}	0.7669^{hij}
S20	0.9965^{fgh}	1.2956^{cde}
S26	0.8457^{ghi}	0.9030^{ghi}
S27	0.9861^{fgh}	1.1958^{def}
S28	0.4640^{kl}	0.5289^{jk}
S29	2.2776^{b}	2.6608^{a}
S30	1.2774^{cde}	1.3379^{cde}
S31	1.4444^{dc}	1.5138^{c}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

Most of the *Ps. aeruginosa* combinations produced either failed to produce a biofilm or produced a weak biofilm. However, S8 (*Ps. aeruginosa, E. coli, E. faecalis, S.* Agona and *S. simulans*) without *C. jejuni* combination was a moderate biofilm producer (Fig 4).There was no significant difference among some of the weak biofilm producers at P< 0.05 (Table 11).

All of the weak biofilm producers had a similar BFI. Even though biofilm produced by the S22 (*Ps. aeruginosa* and *E. faecalis*) combination showed some enhancement in biofilm formation with *C. jejuni,* they were still weak biofilm producers. Although the *Ps. aeruginosa/C. jejuni* combinations often failed to produce a biofilm, the turbidity of the wells in the microtitre plate indicated that microbial growth had occurred. *Ps. aeruginosa* have been shown to have an inhibitory effect on microorganisms *in vitro,* however, no studies have been done on the inhibitory effect of *Ps. aeruginosa* in biofilms (65, 132).

E. faecalis may play a role in the enhancement of biofilm formation by *Ps. aeruginosa,* as when *E. faecalis* was added to *Ps. aeruginosa* combinations weak biofilm production was observed (Fig 4). *C. jejuni* appeared to have some effect in two of these combinations, S8 (*Ps. aerouginosa, E. coli, E. faecalis, S.* Agona, and *S. simulans*), and S25 (*Ps. aerouginosa, E. coli,* and *E. faecalis*). *C. jejuni* appeared to reduce the biofilm formation in S8 while increase the biofilm formation in S25. However, there was no significant difference between the *Ps. aeruginosa* combinations with and without *C. jejuni* (Table 11).

Combinations without *Ps. aeruginosa* often resulted in more biofilm formation except the S17 (*E. coli* and *S.* Agona) combination (Fig 5). *Ps. aeruginosa* used in this study showed an inhibition of biofilm formation. However, this study was in disagreement with Reeser *et.al.,* (2007) who suggested that bacterial culture supernatant fluids of *Ps. aeruginosa* may enhance *C. jejuni* biofilm (128). The results in the present study suggest *Ps. aeruginosa* cells may have a role in inhibition and detachment of other microorganisms besides *C. jejuni.*

6.3.4 Combination with and without *Escherichia coli*

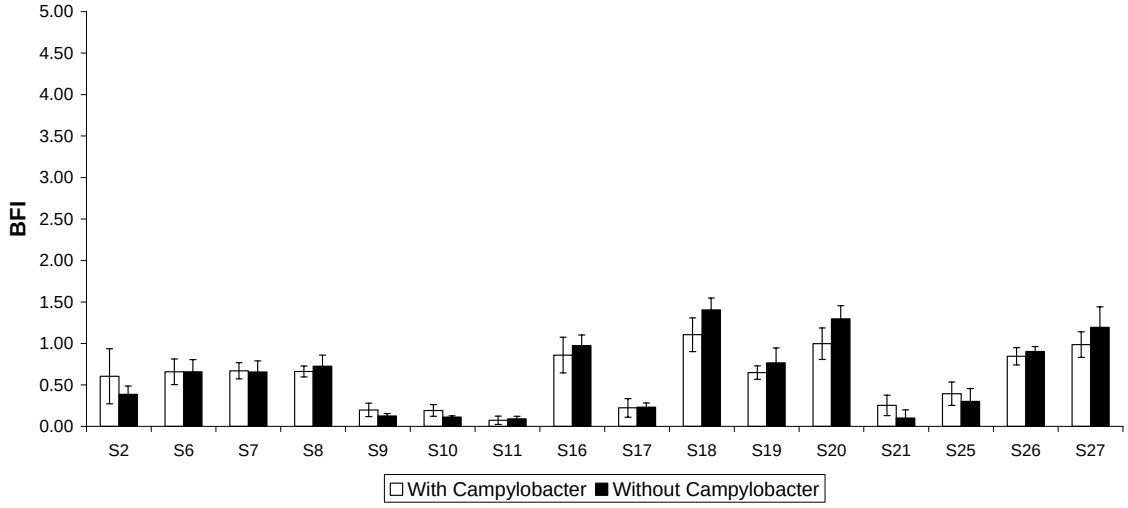

Figure 6 Biofilm formation on polystyrene microtitre plates by different combinations with *Escherichia coli* with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 13 Biofilm formation index (BFI) of combinations with *Escherichia coli*

	With *Campylobacter jejuni* (BFI)	Without *Campylobacter jejuni* (BFI)
S2	0.6044^{ij}	0.3873^{jk}
S6	0.6585^{hi}	0.6590^{hi}
S7	0.6703^{hi}	0.6574^{hi}
S8	0.6626^{hi}	0.7254^{ghi}
S9	0.1988^{klm}	0.1270^{lm}
S10	0.1912^{klm}	0.1121^{lm}
S11	0.0745^{m}	0.0926^{lm}
S16	0.8596^{efgh}	0.9748^{def}
S17	0.2235^{klm}	0.2336^{klm}
S18	1.1046^{bcd}	1.4047^{a}
S19	0.6492^{hi}	0.7669^{fghi}
S20	0.9965^{cde}	1.2956^{ab}
S21	0.2535^{klm}	0.1014^{lm}
S25	0.3944^{jk}	0.3026^{kl}
S26	0.8457^{efgh}	0.9030^{defg}
S27	0.9861^{cdef}	1.1958^{abc}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

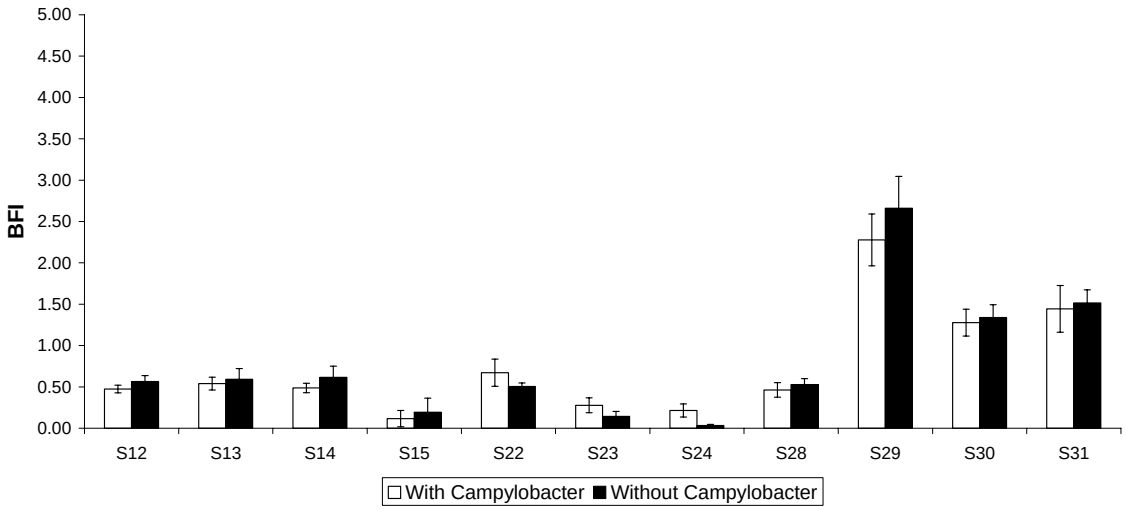

Figure 7 Biofilm formation on polystyrene microtitre plates by different combinations without *Escherichia coli* with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Experiments were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 14 Biofilm formation index (BFI) of combinations without *Escherichia coli*

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S12	0.4748^{ef}	0.5654^{e}
S13	0.5400^{e}	0.5919^{e}
S14	0.4874^{ef}	0.6146^{e}
S15	0.1162^{gh}	0.1940^{gh}
S22	0.6723^{e}	0.5054^{ef}
S23	0.2778^{fg}	0.1440^{gh}
S24	0.2157^{gh}	0.0325^{h}
S28	0.4640^{ef}	0.5289^{e}
S29	2.2777^{b}	2.6608^{a}
S30	1.2774^{d}	1.3379^{cd}
S31	1.4444^{cd}	1.5138^{c}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

E. coli had the ability to form biofilm in monoculture as well as in certain mixed-microbial combinations (Fig 6). *E. coli* in this study showed some reduction in biofilm formation in mixed-microbial cultures. However, the comparisons of the BFI for the *E. coli* combinations with and without *C. jejuni* were similar. Five out of the six none biofilm producers were observed to have *Ps. aeruginosa* in the combination, thus, *Ps. aeruginosa* appeared to inhibit the biofilm formation by *E. coli.* This may due to the inhibitory effects reported for *Ps. aeruginosa* (65, 132).

C. jejuni seemed to have the ability to enhance the biofilm formation by *E. coli* when these two microorganisms were grown together (Fig 6). However, there was no significant difference between the monoculture of *E. coli* and combination of *E. coli* and *C. jejuni* at $P < 0.05$ when all *E. coli* combinations were compared (Table 13).

Two of the *E. coli* combinations, S18 (E. *coli* and *S. simulans*) and S20 (*E. coli, E. faecalis* and *S. simulans*) appeared to reduce biofilm formation when they were inoculated with *C. jejuni*, even though these two *E. coli* combinations showed strong formation of biofilm.

Six of the *E. coli* combinations without *C. jejuni* were not able to form biofilms. These six combinations were S9 (*Ps. aerouginosa, E. coli,* and *S.* Agona), S10 (*Ps. aerouginosa, E. coli, S.* Agona, and *S. simulans*), S11 (*Ps. aerouginosa, E. coli,* and *S. simulans*), S17 (*E. coli,* and *S.* Agona), S21 (*E. coli,* and *S.* Agona), and S25 (*Ps. aeruginosa, E. coli,* and *E. faecalis*). However, S25 with *C. jejuni* appeared to be a weak biofilm producer. There was no significant difference between S25 with and without *C. jejuni* ($P<0.05$).

All except three of the combinations without *E. coli* S15 (*Ps. aerouginosa, S.* Agona, and *S. simulans*), S23 (*Ps. aerouginosa*, and *S.* Agona) and S24 (*Ps. aerouginosa*, and *S. simulans*) (Fig 7) had the ability to form biofilm. *C. jejuni* did not appear to have any effect on biofilm formation by combinations without *E. coli* except the S29 (*E. faecalis*, and *S. simulans*) combination (Table 14). Although *C. jejuni* appeared to significantly reduce biofilm formation by S29 ($P<0.05$), both the S29 combination with and without *C. jejuni* were strong biofilm producers.

6.3.5 Combination with and without *Enterococcus faecalis*

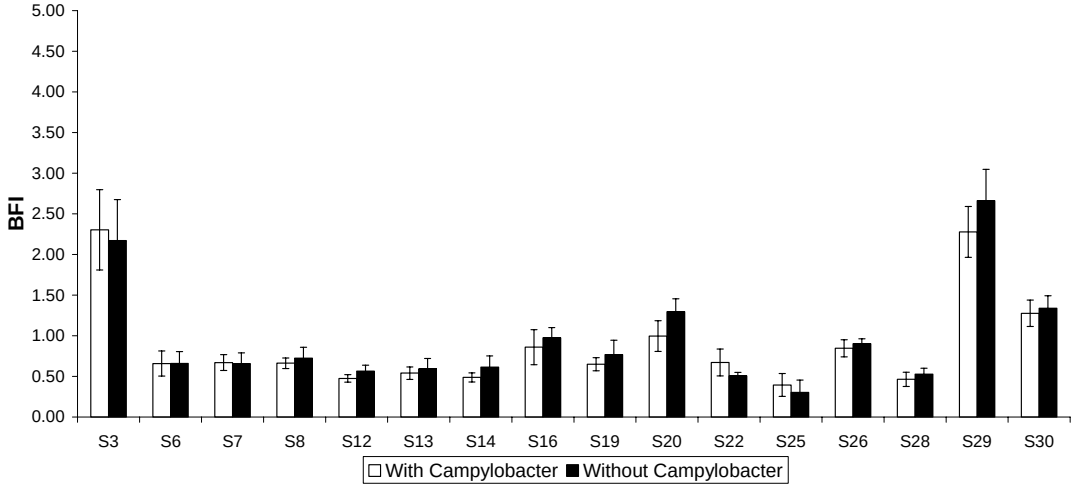

Figure 8 Biofilm formation on polystyrene microtitre plates by different combinations with *Enterococcus faecalis* with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 15 Biofilm formation index (BFI) of combinations with *Enterococcus faecalis*

	With *Campylobacter jejuni* (BFI)	Without *Campylobacter jejuni* (BFI)
S3	2.3029^{b}	2.1715^{b}
S6	0.6585^{fghij}	0.6590^{fghij}
S7	0.6703^{fghij}	0.6574^{fghij}
S8	0.6626^{fghij}	0.7254^{efghi}
S12	0.4749^{hijk}	0.5655^{ghijk}
S13	0.5400^{hijk}	0.5919^{ghijk}
S14	0.4874^{hijk}	0.6146^{fghij}
S16	0.8596^{efg}	0.9748^{e}
S19	0.6492^{fghij}	0.7669^{efgh}
S20	0.9965^{de}	1.2956^{cd}
S22	0.6723^{fghij}	0.5054^{hijk}
S25	0.3944^{jk}	0.3026^{k}
S26	0.8457^{efg}	0.9030^{ef}
S28	0.4640^{ijk}	0.5289^{hijk}
S29	2.2777^{b}	2.6608^{a}
S30	1.2774^{dc}	1.3379^{c}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

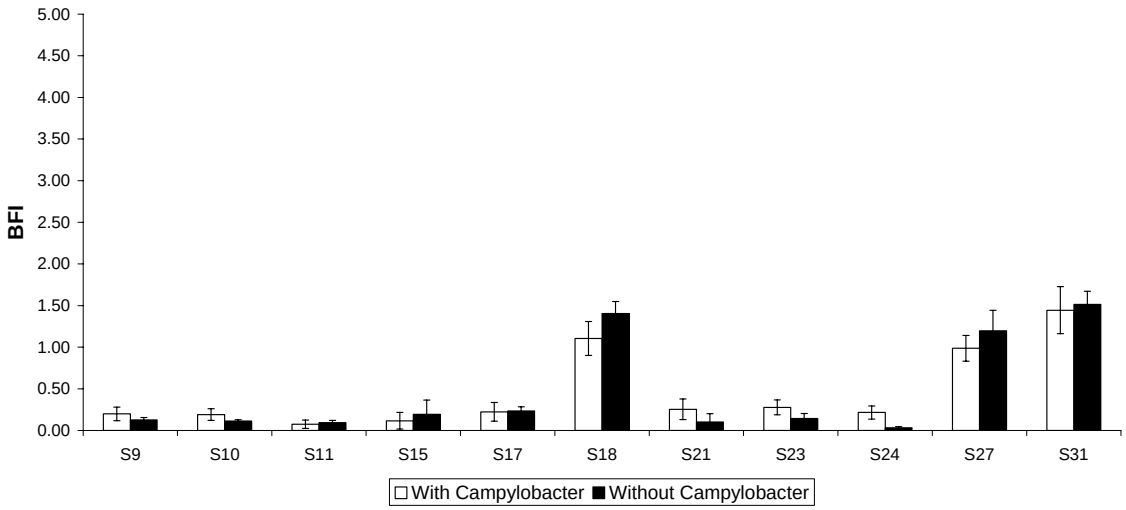

Figure 9 Biofilm formation on polystyrene microtitre plates by different combinations without *Enterococcus faecalis* with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 16 Biofilm formation index (BFI) of combinations without *Enterococcus faecalis*

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S9	0.1988^{def}	0.1270^{def}
S10	0.1912^{def}	0.1121^{def}
S11	0.0745^{ef}	0.0926^{def}
S15	0.1162^{def}	0.1940^{def}
S17	0.2235^{def}	0.2336^{de}
S18	1.1046^{bc}	1.4047^{a}
S21	0.2535^{de}	0.1014^{def}
S23	0.2778^{d}	0.1440^{def}
S24	0.2157^{def}	0.0325^{f}
S27	0.9861^{c}	1.1958^{b}
S31	1.4444^{a}	1.5138^{a}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

Most of the *E. faecalis* combinations had the ability to form biofilm except for the S25 *(Ps. aeruginosa, E. coli,* and *E. faecalis*) combination without *C. jejuni* (Fig 8). Ten of the 16 combinations with *C. jejuni* were weak biofilm producers. However, two of the combinations of the weak biofilm producers, S8 (*Ps. aeruginosa, E. coli, E. faecalis, S.* Agona, and *S. simulans*) and S19 (*E. coli, E. faecalis,* and *S.* Agona) were moderate biofilm producers when the combinations were grown without *C. jejuni* (Fig 8). The S29 (*E. faecalis,* and *S. simulans*) combination had the highest BFI out of all the *E. faecalis* combinations. However, *C. jejuni* showed a significant inhibitory effect in the combination at $P<0.05$.

Strong biofilm formation was observed in S3 (*E. faecalis*), S20 (*E. coli, E. faecalis,* and *S. simulans*), S29 (*E. faecalis,* and *S. simulans*) and S30 (*E. faecalis, S.* Agona, and *S. simulans*) combinations. The BFI for these combinations with and without *C. jejuni* were similar. However, the S20 combination with *C. jejuni* was a moderate biofilm producer. It appeared that there was no significant difference at $P<0.05$ when comparing the other *E. faecalis* combinations (Table 15), however, there was significant difference at $P<0.05$ in biofilm formation between S20 combinations with and without *C. jejuni* (Table 10).

Two of the *E. faecalis* combinations with and without *C. jejuni* were moderate biofilm producers. These two combinations, S16 (*E. coli,* and *E. faecalis*), and S26 (*E. coli, E. faecalis, S.* Agona, and *S. simulans*) did not show any difference in the presence or absence of *C. jejuni.*

In general, there was no significant difference among *E. faecalis* combinations with and without *C. jejuni* at $P<0.05$, even though there was some observed effect with some of the combinations (Table 15). *E. faecalis* may play crucial role in the overall biofilm formation in this study, as combinations without *E. faecalis* did not produce biofilm except for S18 (*E. coli* and *S. simulans*) , S27 (*E. coli ,S.* Agona, *S. simulans*), and S31 (*S.* Agona and *S. simulans*) (Fig 9). These three combinations had *S. simulans*, which may also play a key role in biofilm formation. However, these combinations had a reduced BFI when they were grown with *C. jejuni.*

6.3.6 Combination with and without *Salmonella* Agona

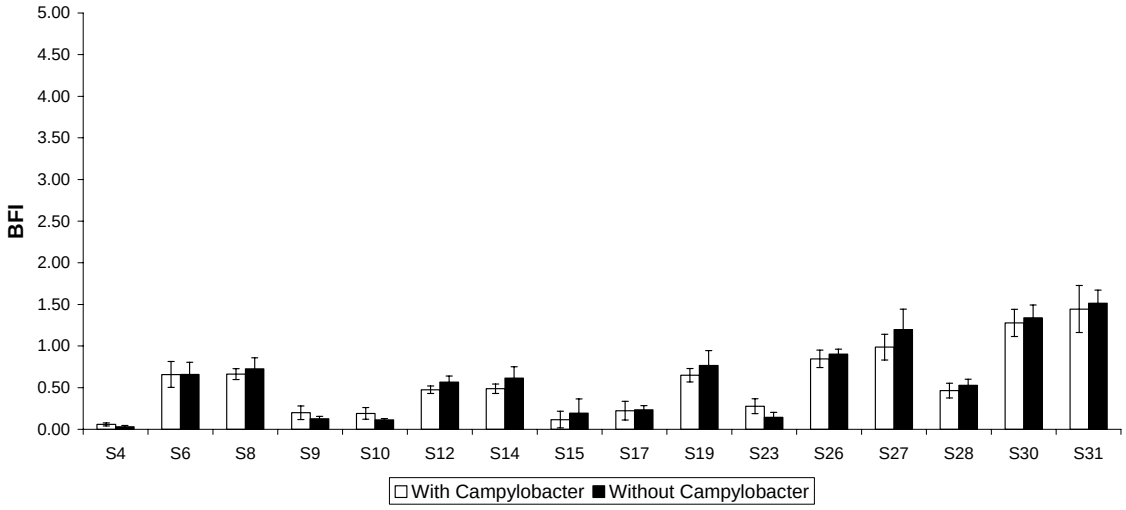

Figure 10 Biofilm formation on polystyrene microtitre plates by different combinations with *Salmonella* Agona with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 17 Biofilm formation index (BFI) of combinations with *Salmonella* Agona

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S4	0.0584^{mn}	0.0318^{n}
S6	0.6585^{fghi}	0.6590^{fghi}
S8	0.6626^{fghi}	0.7254^{efgh}
S9	0.1988^{mn}	0.1270^{lmn}
S10	0.1912^{lmn}	0.1121^{lmn}
S12	0.4749^{ij}	0.5655^{hij}
S14	0.4874^{ij}	0.6146^{ghij}
S15	0.1162^{lmn}	0.1940^{lmn}
S17	0.2235^{lm}	0.2336^{lm}
S19	0.6492^{ghij}	0.7669^{efg}
S23	0.2778^{kl}	0.1440^{lmn}
S26	0.8457^{def}	0.9030^{de}
S27	0.9861^{d}	1.1958^{c}
S28	0.4640^{ik}	0.5289^{ij}
S30	1.2774^{bc}	1.3379^{abc}
S31	1.4444^{ab}	1.5138^{a}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

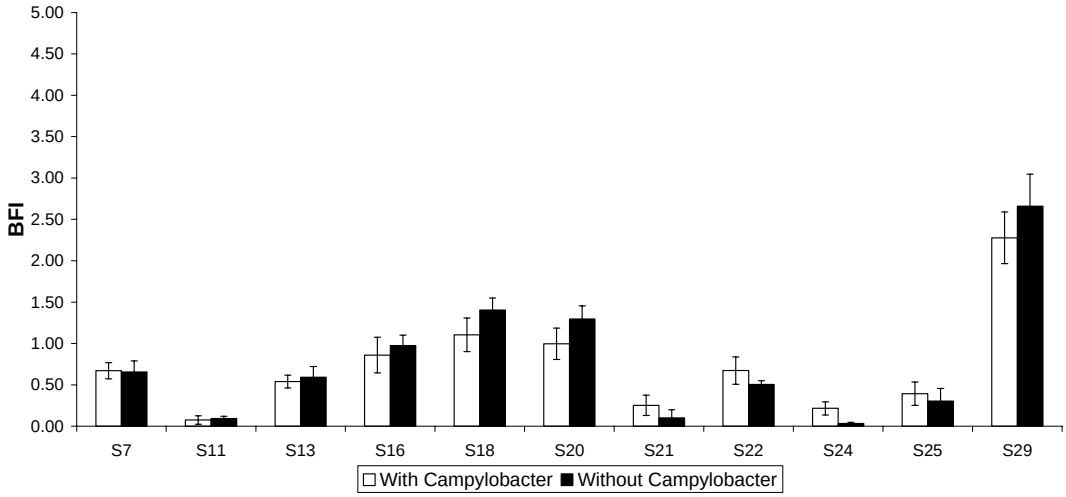

Figure 11 Biofilm formation on polystyrene microtitre plates by different combinations without *Salmonella* Agona with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 18 Biofilm formation index (BFI) of combinations without *Salmonella* Agona

	With *Campylobacter jejuni* (BFI)	Without *Campylobacter jejuni* (BFI)
S7	0.6703^{gh}	0.6574^{gh}
S11	0.0745^{lm}	0.0926^{lm}
S13	0.5400^{hij}	0.5919^{hi}
S16	0.8596^{fg}	0.9748^{ef}
S18	1.1046^{de}	1.4047^{c}
S20	0.9965^{ef}	1.2956^{cd}
S21	0.2535^{klm}	0.1014^{lm}
S22	0.6723^{gh}	0.5054^{hij}
S24	0.2157^{klm}	0.0325^{m}
S25	0.3944^{ijk}	0.3026^{jkl}
S29	2.2777^{b}	2.6608^{a}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

S. Agona used in this study did not produce biofilm on its own although biofilm production increased when *S.* Agona was grown in mixed-microbial culture. However, five of the *S.* Agona combinations still failed to form biofilms (Fig 10). *S. simulans* may play a role in formation of biofilms of *S.* Agona in this study, as strong biofilm formations were observed in S27 (*E. coli, S.* Agona, and *S. simulans*), S30 (*E. faecalis, S.* Agona, and *S. simulans*), and S31 (*S.* Agona, and *S. simulans*) combinations (Fig 10).

S26 (*E. coli, E. faecalis, S.* Agona, and *S. simulans*) combinations with and without *C. jejuni* can form moderate biofilms. S8 (*Ps. aeruginosa, E. coli, E. faecalis, S.* Agona, and *S. simulans*), and S19 (*E. coli, E. faecalis,* and *S.* Agona) combinations without *C. jejuni* were moderate biofilm producers, however, with the presence of *C. jejuni*, the biofilm formation was reduced, but not significantly ($P<0.05$). On the other hand, S27 (*E. coli, S.* Agona, and *S. simulans*) combination without *C. jejuni* produced a significantly stronger biofilm than was seen when *C. jejuni* was present, ($P< 0.05$), suggesting that *C. jejuni* may inhibit biofilm formation in this combination.

Four of the *S.* Agona combinations with and without *C. jejuni* were weak biofilm producers and they had similar BFI's. These combinations were S6 (*Ps. aeruginosa, E. coli, E. faecalis,* and *S.* Agona), S12 (*Ps. aeruginosa, E. faecalis,* and *S.* Agona), S14 (*Ps. aeruginosa, E. faecalis, S.* Agona, and *S. simulans*) and S28 (*E. faecalis,* and *S.* Agona).

Most of the combinations without *S.* Agona had the ability to form biofilm except S11 (*Ps. aeruginosa, E. coli,* and *S. simulans*), S21 (*Ps. aeruginosa,* and *E. coli*) and S24 (*Ps. aeruginosa,* and *S. simulans*) (Fig 11).

Five of the combinations without *S.* Agona showed some changes in biofilm formation when combined with *C. jejuni* (Fig. 11). S22 (*Ps. aeruginosa,* and *E. faecalis*) and S25 (*Ps. aeruginosa, E. coli,* and *E. faecalis*) combinations with *C. jejuni* showed an increase of biofilm production, however, there was no significant difference at $P<0.05$ with S22 and S25 combinations without *C. jejuni*. Both of these combinations also contained *E. faecalis,* which may help to explain the increased BFI.

The following combinations appeared to have a lower BFI in the present of *C. jejuni.* These combinations were S18 (*E. coli,* and *S. simulans*), S20 (*E. coli, E. faecalis,* and *S. simulans*) and S29 (*E. faecalis,* and *S. simulans*) combinations. *C. jejuni* may inhibit the development of biofilms when *C. jejuni* was grown in these combinations.

6.3.7 Combination with and without *Staphylococcus simulans*

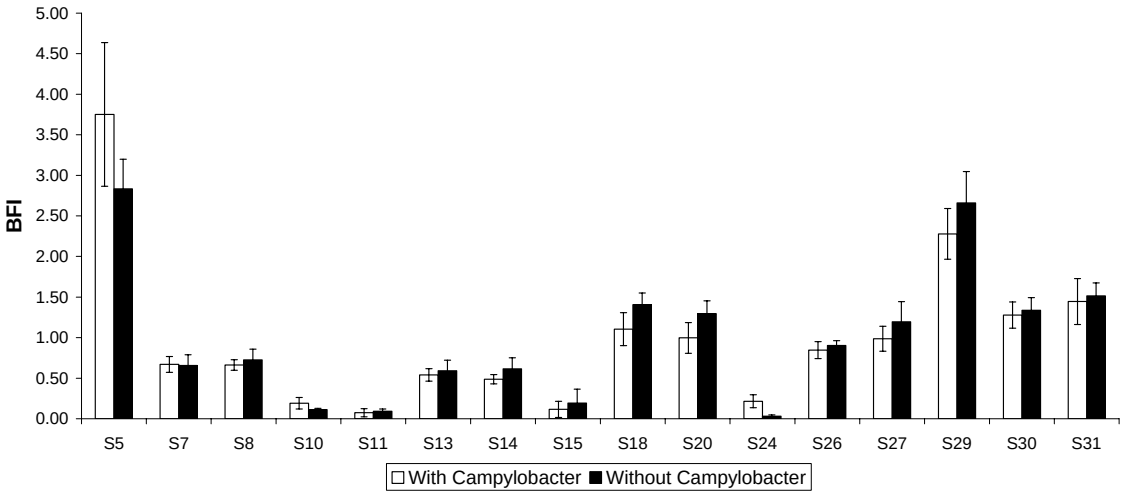

Figure 12 Biofilm formation on polystyrene microtitre plates by different combinations with *Staphylococcus simulans with* gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 19 Biofilm formation index (BFI) of combinations with *Staphylococcus simulans*

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S5	3.7515^{a}	2.8335^{b}
S7	0.6703^{hijk}	0.6574^{hijk}
S8	0.6626^{hijk}	0.7254^{hijk}
S10	0.1912^{lm}	0.1121^{m}
S11	0.0745^{m}	0.0926^{m}
S13	0.5400^{jkl}	0.5919^{ijk}
S14	0.4874^{kl}	0.6146^{ijk}
S15	0.1162^{m}	0.1940^{lm}
S18	1.1046^{efg}	1.4047^{de}
S20	0.9965^{fgh}	1.2956^{def}
S24	0.2157^{lm}	0.0325^{m}
S26	0.8457^{ghij}	0.9030^{ghi}
S27	0.9861^{fgh}	1.1958^{defg}
S29	2.2777^{c}	2.6608^{b}
S30	1.2774^{def}	1.3379^{def}
S31	1.4444^{de}	1.5138^{d}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test).

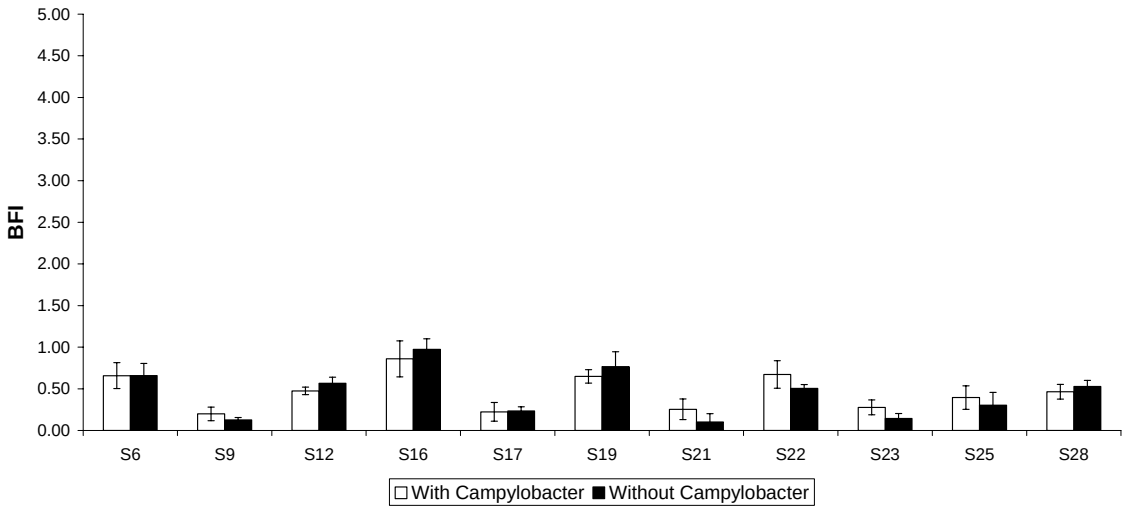

Figure 13 Biofilm formation on polystyrene microtitre plates by different combinations without *Staphylococcus simulans with* gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. In total, there were 16 combinations. Trials were performed on two separate occasions, and error bars represent one standard deviation from the mean.

Table 20 Biofilm formation index (BFI) of combination without *Staphylococcus simulans*

	With *Campylobacter jejuni* (BFI)	**Without *Campylobacter jejuni* (BFI)**
S6	0.6585^{cd}	0.6590^{cd}
S9	0.1988^{hij}	0.1270^{ij}
S12	0.4749^{ef}	0.5655^{de}
S16	0.8596^{ab}	0.9748^{a}
S17	0.2235^{ghij}	0.2336^{ghij}
S19	0.6492^{cd}	0.7669^{bc}
S21	0.2535^{ghij}	0.1014^{j}
S22	0.6723^{cd}	0.5054^{cd}
S23	0.2778^{ghi}	0.1440^{hij}
S25	0.3944^{efg}	0.3026^{fgh}
S28	0.4640^{ef}	0.5289^{de}

Means with the same letters are not significantly different at $P < 0.05$ (Tukey's test)

S. simulans formed biofilm in monoculture as well as in mix culture. *S. simulans* had the highest BFI when it was grown with *C. jejuni*, followed by *S. simulans* in monoculture. *C. jejuni* appeared to enhance the development of biofilm formation by *S. simulans* when they were in a one-to-one combination. However, the effect of this enhancement was reversed when there was more than one other microorganism involved suggesting some complex interactions in the mixed populations.

The following six of the *S. simulans* combinations without *C. jejuni* produced a high BFI: S18 (*E. coli,* and *S. simulans*), S20 (*E. coli, E. faecalis,* and *S. simulans*) S27 (*E. coli, S.* Agona, and *S. simulans*), S29 (*E. faecalis,* and *S. simulans)*, S30 (*E. faecalis, S.* Agona, *and S. simulans*), and S31 (*S.* Agona, and *S. simulans*). However, two of the *S. simulans* combinations were moderate biofilm producers when the *S. simulans* combinations were grown with *C .jejuni,* (S20 and S27). These two *S. simulans* combinations showed no significant difference in biofilm production, with or without *C. jejuni* ($P<0.05$) (Table 19).

The following five *S. simulans* combinations without *C. jejuni* produced a weak or moderate biofilm: S7 (*Ps. aerouginosa, E. coli, E. faecalis,* and *S. simulans*), S8 (*Ps. aerouginosa, E. coli, E. faecalis, S.* Agona, and *S. simulans*), S13 (*Ps. aerouginosa, E. faecalis,* and *S. simulans*), S14 (*Ps. aerouginosa, E. faecalis, S.* Agona, and *S. simulans*), and S26 (*E. coli, E. faecalis, S.* Agona, *and S. simulans*). These *S. simulans* combinations without *C. jejuni* had similar BFI's (Table 19). *C. jejuni* did not seem to have any effect on biofilm production by these combinations.

Overall, *S. simulans* combinations were able to form good biofilm except for S10 *(Ps. aeruginosa, E. coli, S.* Agona, and *S. simulans*), S11 (*Ps. aeruginosa, E. coli,* and *S. simulans*), S15 (*Ps. aeruginosa, S.* Agona, and *S. simulans*) and S24 (*Ps. aeruginosa,* and *S. simulans*) (Fig 12). These S. *simulans* combinations did not appear to be able to form biofilms. *Ps. aeruginosa* may play a role in the inhibition of biofilm production by *S. simulans,* as has been seen in other combinations that have included *Ps. aeruginosa.*

Combinations without *S. simulans* were unable to form good biofilms. S16 (*E. coli*, and *E. faecalis*) combinations with and without *C. jejuni* were able to form moderate biofilm, while others either produced weak or no biofilm (Fig 13). *C. jejuni* may inhibit the production of biofilm in S19 (*E. coli, E. faecalis,* and *S.* Agona) combinations, as S19 without *C. jejuni* was a moderate biofilm producer while in S19 with *C. jejuni,* biofilm formation was significantly reduced ($P < 0.05$). *S. simulans* may play an important role in the overall biofilm production in this study.

6.3.8 Comparison of overall biofilm formation by specific types of microorganisms

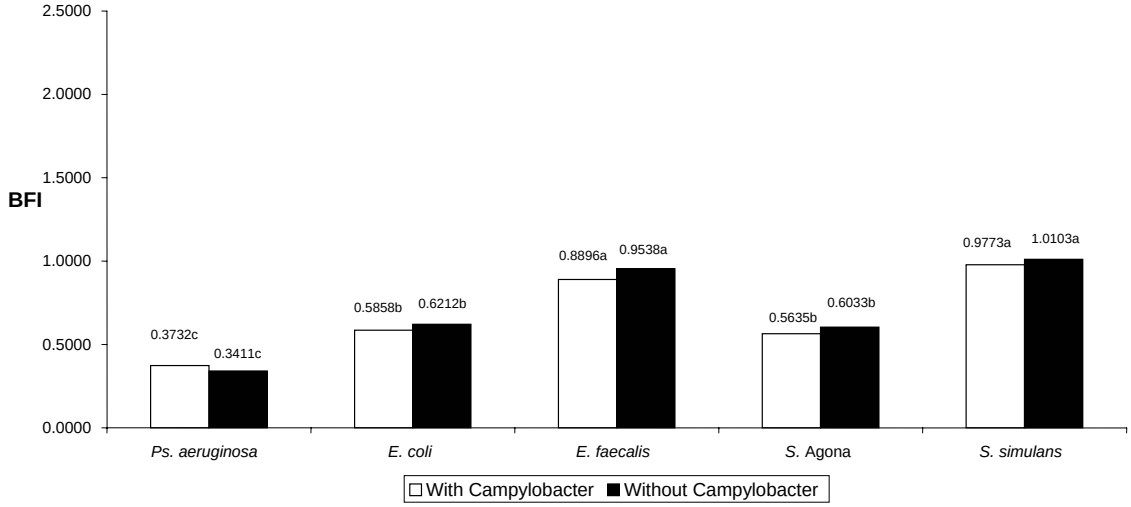

Figure 14 Comparison of total BFI on polystyrene microtitre plates for test combinations with the specific microorganisms listed on the X-axis, with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. Trials were performed on two separate occasions. P <0.05 (Tukey's test).

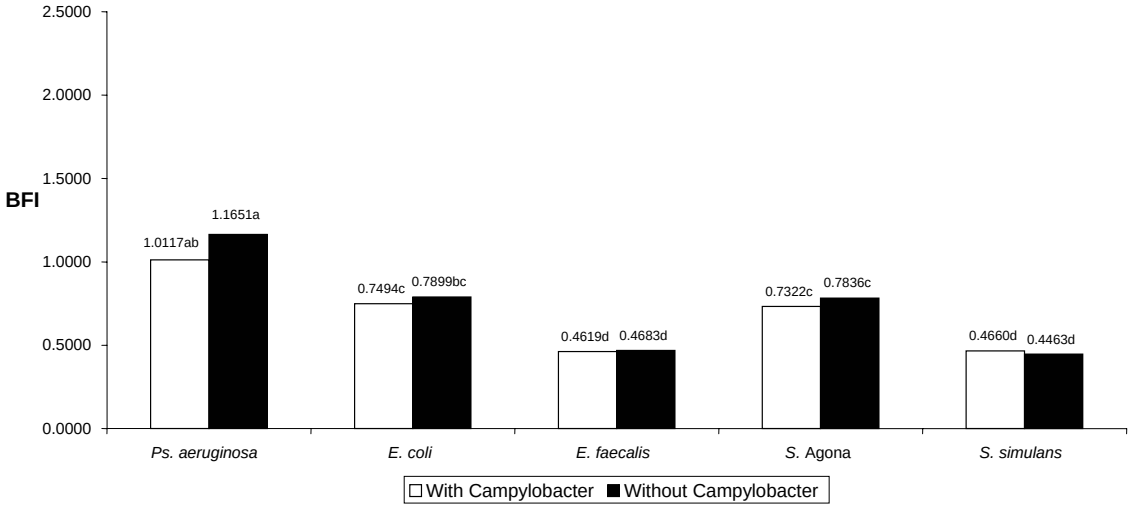

Figure 15 Comparison of total BFI on polystyrene microtitre plates for test combinations without the specific microorganisms listed on the X-axis, with gentle swirling (30 rpm) at 37°C for 72 h under microaerophilic conditions. Trials were performed on two separate occasions. P <0.05 (Tukey's test)

In general, microorganisms used in this study were able to form biofilm either alone or in combinations with other microorganisms (Fig 14). High biofilm formation was observed in the presence of *E. faecalis* and *S. simulans*. Both of these microorganisms were moderate biofilm producers. *C. jejuni* did not show any effect on the biofilm production by *E. faecalis* or *S. simulans* combinations.

The other three microorganisms (*Ps. aeruginosa, E. coli* and *S.* Agona) used in this study were weak biofilm producers. There were no differences observed between in the biofilm formation of *E. coli* and *S.* Agona. *Ps. aeruginosa* was the weakest biofilm producer. *C. jejuni* did not seem to have any influence on the biofilm formation by these microorganisms, as the BFI's with and without *C. jejuni* were similar.

Ps. aeruginosa, E. faecalis, and S. simulans had the most effect on biofilm formation in this study. Combinations without *Ps. aeruginosa* produced high BFI's compared with the combinations containing *Ps.* aeruginosa (Fig 15). *Ps aeruginosa* seemed to inhibit the biofilm formation in this study, as low BFI's were observed when *Ps. aeruginosa* was incorporated into the combinations. The combinations without *E. faecalis* and *S. simulans* were the weakest biofilm producers. *E. faecalis* and *S. simulans* appeared to play a major role in biofilm formation in this study, as in any combinations with these two microorganisms tended to have high BFI's.

6.3.9 Enumeration and cell recovery of *C. jejuni* from biofilm

Table 21 Enumeration of *C. jejuni* from biofilm

Sample	Individual/Combinations of microorganisms	Log_{10} c.f.u/ mL	std dev
S1	*Ps. aerouginosa*	0	0
S2	*E. coli*	1.716^{hg}	0.323
S3	*E. faecalis*	3.403^{a}	0.546
S4	*S.* Agona	2.038^{efg}	0.497
S5	*S. simulans*	1.805^{fgh}	0.496
S6	*Ps. aerouginosa, E. coli, E. faecalis, S.* Agona	0.500^{ij}	0.535
S7	*Ps. aerouginosa, E. coli, E. faecalis, S. simulans*	0	0
S8	*Ps. aerouginosa, E. coli, E. faecalis, S.* Agona*, S. simulans*	0.125^{j}	0.354
S9	*Ps. aerouginosa, E. coli, S.* Agona	0	0
S10	*Ps. aerouginosa, E. coli, S.* Agona*, S. simulans*	0.489^{ij}	0.905
S11	*Ps. aerouginosa, E. coli, S. simulans*	0.750^{I}	1.035
S12	*Ps. aerouginosa, E. faecalis, S.* Agona	0	0
S13	*Ps. aerouginosa, E. faecalis, S. simulans*	0	0
S14	*Ps. aerouginosa, E. faecalis, S.* Agona*, S. simulans*	0	0
S15	*Ps. aerouginosa, S.* Agona*, S. simulans*	0	0
S16	*E. coli, E. faecalis*	0	0
S17	*E. coli, S.* Agona	1.614^{h}	0.394
S18	*E. coli, S. simulans*	2.069^{efg}	0.103
S19	*E. coli, E. faecalis, S.* Agona	0.700^{i}	0.593
S20	*E. coli, E. faecalis, S. simulans*	2.301^{cde}	0.256
S21	*Ps. aerouginosa, E. coli*	1.704^{gh}	0.389
S22	*Ps. aerouginosa, E. faecalis*	0	0
S23	*Ps. aerouginosa, S.* Agona	0	0
S24	*Ps. aerouginosa, S. simulans*	0	0
S25	*Ps. aerouginosa, E. coli, E. faecalis*	0.125^{j}	0.354
S26	*E. coli ,E. faecalis, S.* Agona*, S. simulans*	1.625^{h}	0.790
S27	*E. coli ,S.* Agona*, S. simulans*	2.140^{def}	0.186
S28	*E. faecalis, S.* Agona	2.530^{bcd}	0.372
S29	*E. faecalis, S. simulans*	3.421^{a}	0.343
S30	*E. faecalis, S.* Agona*, S. simulans*	2.662^{bc}	0.218
S31	*S.* Agona*, S. simulans*	2.780^{b}	0.269

Means with the same letters are not significantly different at $P < 0.05$ (T- test)
Note 0 log = < 1cfu /mL

C. jejuni was able to be recovered from biofilms in this study (Table 21). The number of cells recovered ranged from 0 log c.f.u/mL to 3.421 log c.f.u/ mL. *C. jejuni* was able to attach and survive in most of the combinations, except for most of the *Ps. aeruginosa* combinations. Although no viable cells were able to be recovered from most of the *Ps. aeruginosa* combinations, *C. jejuni* may be able to survive in these combinations. This is because the recovery method used in this study does not completely recover the cells from the biofilm. In 2003, Mai stated that many of the psychrotrophic spoilage organisms commonly associated with the poultry carcass would reduce the numbers of *C. jejuni* in both broth and agar cultures by as much as 5.8 log c.f.u/mL (39).

The results from the laboratory tests in this study agree with the observation of *Conner et al.,* (2005) in that *C. jejuni* can be isolated from mixed *Pseudomonas* spp. in poultry carcasses, and stating that *Pseudomonas* spp. may reduce *C. jejuni in vitro* (39). In the present study, *C. jejuni* could only be isolated from two of the 16 *Ps. aeruginosa* combinations. This suggests that the chance of isolating *C. jejuni* from environments where *Ps. aeruginosa* is present may be low. However, this may also reflect differences between a laboratory trial and the natural poultry environment.

High numbers of *C. jejuni* were able to be recovered from biofilms formed by *E. faecalis*. This finding was in agreement with Trachoo *et al.,* (2005), which stated that *C. jejuni* might attach onto the biofilm formed by *Enterococcus faecium*. Although *S. simulans* was a strong biofilm producer, the recovery of *C. jejuni* was not as great as from combinations that included *E. faecalis*.

Most of the *S. simulans* combinations and *E. faecalis* combinations were able to harbour *C. jejuni* in the biofilm except a few of the combinations where *Ps. aerouginosa* was present. *Ps. aerouginosa* may reduce the numbers of *C. jejuni* before *C. jejuni* can attach onto the biofilm.

C. jejuni was able to be isolated from S4 even though S4 (*S.* Agona and *C. jejuni*), which did not produce any biofilm. *C. jejuni* may attach onto the wall of the plastic well.

Although *E. coli* was a weak biofilm producer, *C. jejuni* was able to be recovered from the biofilm. This suggested that *C. jejuni* was able to attach and survive in a low biofilm mass. This may help to protect *C. jejuni* from environmental stress.

These results reflect interactions between different species in a biofilm community. The effect of *P. aerouginosa* in reducing biofilm formation and the effect of *E. faecalis and S. simulans* enhancing biofilm formation are interesting findings that may affect the survival of *C. jejuni* in the poultry environment.

Chapter 7 General discussion and Conclusion

Poultry consumption has been increasing since 1975 in New Zealand and it represented has the highest percentage of meat consumed in 2006. Poultry meat is a good source of protein; however, consumption of undercooked poultry has been associated with campylobacteriosis. Campylobacteriosis is a food-borne disease that is caused by *Campylobacter*. *C. jejuni* has been isolated from the poultry products, poultry farms and poultry processing plants. *C. jejuni* can attach and survive in biofilms. Biofilms can be found in the water supply pipes on poultry farms, and on rubber and stainless steel surfaces in poultry processing plant. Biofilms can protect *C. jejuni* from natural environmental stress and chemical agents, and provide a source of contamination of the poultry. This study investigated the biofilm growth *C. jejuni* in controlled mixed-microbial populations.

Biofilm formation by *C. jejuni* varies from strain to strain. Not all the *C. jejuni* strains tested in this study were able to form strong biofilms. Only a few strains were capable of forming good biofilm and this agrees with other studies (85, 128). Although *C. jejuni* were able to form biofilms, the amount of biofilm formed was not as great as that produced by other microorganisms used in the study. The poultry strains of *C. jejuni* did not appear to form biofilm to the same extent as human clinical strains of *C. jejuni*, even though they may have the same MLST. Although there was no connection between biofilm formation and the MLST, the MLST can be used as an indicator in tracking the source of human clinical strains from the environment (98, 100). By tracking the MLST types of *C. jejuni,* control measures can be taken to minimize the source of contamination in the poultry industry.

C. jejuni did not produce a consistent positive or negative effect on biofilm formation in mixed-microbial populations. However, for some specific microbial combinations, there was some effect on biofilm formation. These effects on biofilm formation may be due to quorum sensing activities or antimicrobial or interspecies competition within the biofilm community.

Good biofilm formation was observed in the mixed-microbial populations that included either *Enterococcus faecalis* and/or *Staphylococcus simulans*. These two microorganisms were shown to have good biofilm forming ability when they were grown under microaerophilic conditions suitable for *C. jejuni*. These microorganisms could provide a safe haven for the *C. jejuni* in the poultry environment, as both of these microorganisms originated from poultry source. *C. jejuni* have been found to attach on biofilms produced by *E. faecalis* and *S. simulans* (134, 151, 160). These microorganisms have been found in both the poultry farm and poultry processing plant environments (44, 62, 124). Biofilm formation in the poultry environment may reduce, if these two microorganisms are eradicated. Further studies are required to investigate the relationships among these microorganisms and the means of removing them.

Pseudomonas aeruginosa appeared to have an inhibitory effect on the production of biofilm. Most of the mixed-microbial populations that included *Ps. aeruginosa* were either unable to form biofilms or formed weak biofilms. *Pseudomonas* spp. are usually associated with food spoilage at low temperatures (45, 154). *Ps. aeruginosa* is usually a strong biofilm producer, however, in this study; the production of biofilm was low, which may be due to the growth conditions – in particular the microaerophilic conditions needed to support the growth of *C. jejuni*. Although *Ps. aeruginosa* appeared to have the ability to grow in microaerophilic conditions, and is known to survive in anaerobic conditions by pyruvate fermentation (50), *Ps. aeruginosa* did not appear to show any biofilm formation under these conditions.

The low biofilm formation in *Ps. aeruginosa* mixed-microbial populations may be due to the competitive environment and limited nutrient availability that may reduce the number of cells attaching, and forming biofilm.

Ps. aeruginosa seemed to inhibit *C. jejuni*, as in most of the mixed-microbial populations that included *Ps. aeruginosa*, *C. jejuni* was unable to be recovered. *Ps. aeruginosa* has been reported to inhibit *E. coli* and other microorganisms (65, 132). However, there was a study stating that *Ps. aeruginosa* did not significantly reduce the number of *C. jejuni* cells in poultry meat, which may be due to the complexity of natural microorganisms present (39).

Ps. aeruginosa has the ability to inhibit other microorganisms in planktonic populations. This inhibitory effect may be due to the production of siderophores or conversion of glucose to gluconate by *Pseudomonas* spp (39, 65, 132).This effect may not be obvious in the poultry environment, as there are many types of microorganisms in the natural poultry environment, which may influence the growth and biofilm formation. However, this study has shown that *Ps. aeruginosa* may inhibit or reduce the biofilm formation by five other types of microorganisms that are associated with the poultry environment.

C. jejuni were able to be recovered from biofilms of most of the mixed-microbial populations. *C. jejuni* have the ability to attach and survive in the biofilms, even though the number of cells recovered varied between the different mixed-microbial populations (77, 134, 152, 153). *C. jejuni* seemed to be able to attach and survive well in a mixed-microbial population of *E. faecalis* and *S. simulans* compared with other mixed-microbial populations. Both *E. faecalis* and *S. simulans* were strong biofilm producers on their own, which may explain the high numbers of *C. jejuni* likely to be associated with these biofilms. The presence of *E. faecalis* and/or *S. simulans* in the poultry environment may be a useful indicator of *C. jejuni* contamination.

C. jejuni may have survived in the mixed-microbial populations in low numbers that may not be detectable by the swabbing method used in this study. Preliminary trials demonstrated the limitation of using swabbing method to recover *C. jejuni* from biofilms in that not all the biofilm cells were able to be recovered. The actual numbers of cells recovered will be less than the numbers present in the biofilm, however relative differences should still be detected.

The presence of *C. jejuni* in biofilms can be a problem to the poultry industry. This is because biofilms can protect *C. jejuni* from environmental physical and chemicals stress, with the persistent biofilm providing a source of contamination of poultry meat. (41, 47, 67, 110). In this study, *C. jejuni* survived in wide range of mixed-microbial populations. However, only two of mixed-microbial populations where *Ps. aeruginosa* was present were able to harbour *C. jejuni*. It would be interesting to explore this further and determine the mechanism of inhibition and the potential of using this as a natural control measure for *C. jejuni* colonisation.

In conclusion, *C. jejuni* strains in monoculture have been shown to attach to an abiotic surface and form biofilms to various degrees, thus potentially enhancing their survivability in the poultry environment. *C. jejuni* were shown to have the ability to attach and survive in mixed-microbial populations, however, this does not represent the overall microbial population, as only few of the microorganisms selected were used in the study.

Biofilm formation may play a role in the epidemiology of *C. jejuni* infections. Our study should therefore be extended to examine more strains from human infections, bird populations, and environmental samples from poultry farms and poultry processing plants. The goal should be to establish control measures against the formation of biofilms containing *C. jejuni* in poultry farms and poultry processing plants in New Zealand to reduce the reservoir of contamination and thus reduce the incidence of campylobacteriosis.

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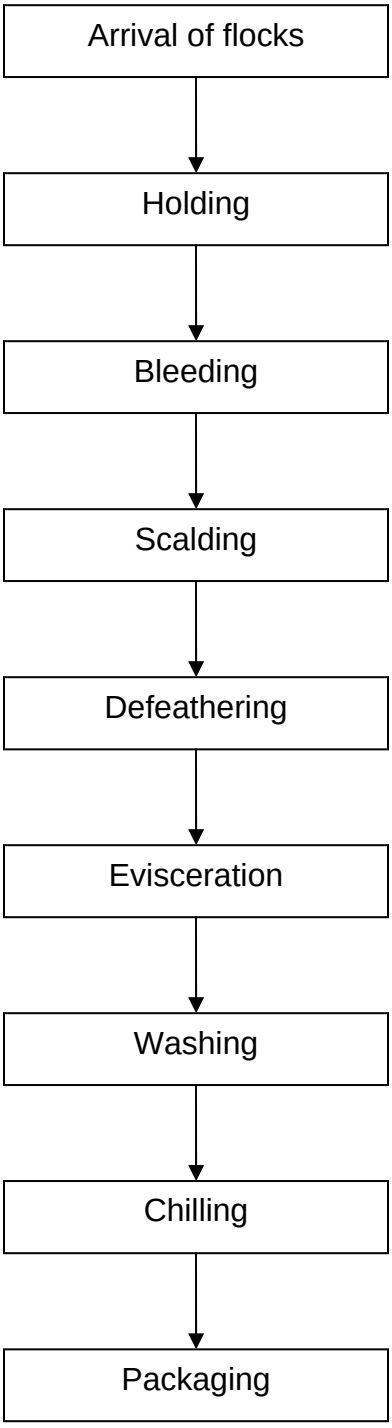
Appendix 1



A simple flow diagram of poultry processing