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

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ABSTRACT

This study was to screen the ability of biofilm formation by *Campylobacter jejuni* strains found in New Zealand, and investigate the biofilm growth of *C. jejuni* in a controlled mixed-microbial population that includes five different bacteria. The ability of *C. jejuni* to form a biofilm in monoculture and mixed-microbial populations was measured in a laboratory assay using a microtiter plate screening assay. The optical density of the biofilm and cell growth from mixed-microbial populations was converted to a Biofilm Formation Index (BFI). This index was used to standardize the biofilm formation in the mixed-microbial populations. High BFI was observed for *Enterococcus faecalis* (2.30) and *Staphylococcus simulans* (3.75) when they were grown with *C. jejuni* multilocus sequence type ST-474: a dominant poultry and human-associated type in New Zealand. *C. jejuni* cells were recovered from most of the biofilms containing *E. faecalis* and/or *S. simulans*. These results suggest that *E. faecalis* and *S. simulans* may play a role in biofilm formation in the poultry environment as both of these microorganisms are found in poultry processing environments and were able to form a biofilm in association with *C. jejuni* under microaerobic conditions. Understanding the relationships among *C. jejuni*, *E. faecalis* and *S. simulans* in poultry processing plants and farms may help in the design of strategies to reduce the reservoir of contamination of these bacteria and reduce the incidence of campylobacteriosis.

1. Introduction

Campylobacteriosis remains a major problem in New Zealand, despite recent improvements in poultry production and a decline in human cases (Mullner et al., 2009). There were 12,776 reported cases of campylobacteriosis in 2007: a year in which campylobacteriosis accounted for 65.9% of all notified diseases (Population and Environmental Health Group and Institute of Environmental Science and Research Limited, 2008). New Zealand has a higher reported rate of campylobacteriosis than other developed countries.

The rate of campylobacteriosis has 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. Cases of campylobacteriosis have been estimated to cost New Zealand $40,136,000 annually, 73% of the total economic cost of food-borne infectious intestinal diseases (Scott et al., 2000).

Campylobacter species can be further differentiated into definitive types by multilocus sequence type (MLST), a method of identifying microbial isolates by their nucleotide sequence data (Maiden et al., 1998). The most commonly identified *C. jejuni* MLST in the world include members of clonal complexes ST-45 and ST-48. However, it is estimated that approximately one third of cases in New Zealand are caused by ST-474, a member of clonal complex ST-48 (McTavish et al., 2008; Mullner et al., 2009). This ST is unique because it is commonly found in New Zealand human cases but rarely found in other countries, and has been isolated from poultry products in New Zealand.

C. jejuni is the predominant species among other *Campylobacter* species in commercial broiler farming (Saenz et al., 2000; Avrain et al., 2003). *Campylobacter* species can survive on fresh and frozen retail poultry products (Istre et al., 1984; Atterbury et al., 2003). The intestinal tract of poultry is a good reservoir for *Campylobacter* species as it is commensal in poultry, with the number of cells in the intestinal tract reaching 10^4 to 10^8 cfu/g (Beery et al., 1988). The main route for *Campylobacter* species transmission in the poultry industry is by horizontal transmission (Atanassova and Ring, 1999).

Biofilms can be found in virtually all aquatic ecosystems that can support microbial growth such as industrial or potable water-system piping (Denyer et al., 1993; Sutherland, 2001; Donlan, 2002). Biofilm is an assemblage of microbial cells that are associated with a surface and enclosed in a matrix of primarily polysaccharide materials, and may contain non-cellular materials which are incorporated into the biofilms from the surrounding environment in which the biofilms are formed (Donlan, 2002).

Campylobacter spp. has been found to attach to biofilms, which are found in the watering supplies and plumbing system of animal-husbandry facilities and animal-processing plants (Buswell et al., 1998). These biofilms are a haven for pathogenic and non-pathogenic

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microorganisms, as biofilms can protect the microorganisms from environmental stress and antimicrobial agents that are used in sanitizing the animal-husbandry facilities and animal-processing plants (Arnold and Silvers, 2000; Trachoo and Brooks, 2005). Biofilms may be a source of contamination with *C. jejuni* in the poultry industry.

Most biofilms are composed of mixtures of microorganisms which lead to interspecies and intraspecies interactions, and to the general complexity of the macromolecular mixture (Sutherland, 2001). Often these interactions result in enhancing the resistance of the microbial population to environmental stress. In the poultry industry, interactions between specific environmental isolates and *C. jejuni* in a biofilm may have implications for the survival of *C. jejuni*, and may be a potential source of contamination in the poultry processing environment.

In this study, a method was used to screen the biofilm formation by different *C. jejuni* strains. The strong biofilm former of *C. jejuni* from the screening study was examined for its ability to survive in controlled mixed-microbial populations, as previous studies have reported the interaction between *C. jejuni* and a strain of microorganism, or a general population of bacteria isolated from the poultry environment. This study looked at the interaction of *C. jejuni* in a controlled mixed-microbial population made up of five different types of bacteria that may be found in a poultry environment.

2. Materials and methods

2.1. Bacterial strains and media

In total, 20 different strains were used for the screening of biofilm formation by *C. jejuni* (Table 1). The bacteria were grown on Columbia blood sheep agar (Fort Richard, New Zealand) at 42 °C for 48 h in a microaerobic incubator (N_2, 85%; O_2, 5%; CO_2, 10%). Five bacterial species that can be associated with the poultry environment (Table 2) were grown on Columbia blood sheep agar at 37 °C for 24 h. All these strains were obtained from the Hopkirk Research Institute at Palmerston North (New Zealand).

The five specific microorganisms were mixed into controlled mixed-microbial populations. In total, there were 31 different experiments including 26 controlled mixed-microbial populations 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 summarized in Table 3.

Table 1
C. jejuni (20 strains) screened for the ability to form biofilm.

Sequence type	Source
ST-45	Poultry
ST-474	Poultry
ST-520	Poultry
ST-25	Poultry
ST-48	Poultry
ST-52	Poultry
ST-227	Poultry
ST-1517	Poultry
ST-190	Poultry
ST-257	Poultry
ST-2345	Poultry
ST-3609	Poultry
ST-53	Poultry
ST-21	Poultry
ST-61	Human
ST-474	Human
ST-45	Human
ST-48	Human
ST-190	Human
ST-177	Water

Table 2
Five specific bacteria strains used in studying the effect on biofilm formation by *C. jejuni* in a controlled mixed-microbial population study.

ID number	Source	Microorganism
En35	Poultry	*Enterococcus faecalis*
EC	Poultry	*Escherichia coli*
Ps13	Water bath	*Pseudomonas aeruginosa*
S62	Poultry	*Salmonella agona*
ST	Poultry	*Staphylococcus simulans*

2.2. Screening strains of Campylobacter jejuni for the ability to form a biofilm

The microtiter plate biofilm screening assay was slightly modified by using 98% ethanol and the optical density was determined at 595 nm (O'Toole and Kolter, 1998) and used to quantify biofilm formation by different sequence types of *C. jejuni*. *C. jejuni* strains were grown overnight in Mueller–Hinton broth (MHB; Difco) at 42 °C under microaerobic conditions (N_2, 85%; O_2, 5%; CO_2, 10%). Overnight cultures were gently shaken and 1 mL volumes inoculated into 5 mL of fresh MHB. The inoculated broths were gently shaken for 30 s, and 0.2 mL volumes transferred into 12 wells of a sterile polystyrene microtiter plate (BD Falcon, USA). Each plate included 12 control wells comprising 0.2 mL of uninoculated MHB. Even though most of the surfaces in the poultry environment are stainless steel, polystyrene microtiter plates were used in the study because they were convenient for a biofilm assay and the focus was on the ability of *C. jejuni* surviving in controlled mixed-microbial populations on a standardized substrate.

2.3. Biofilm formation by Campylobacter jejuni in controlled mixed-microbial populations

The five non-campylobacter bacteria were grown overnight in Mueller–Hinton broth (MHB; Difco) at 37 °C. The overnight broth

Table 3
Combinations of microorganisms used in studying biofilm formation by *C. jejuni* in controlled individual and mixed-microbial populations as negative control.

Id number	Individual/combinations of microorganisms
S1	*Ps. aerouginosa*
S2	*E. coli*
S3	*E. faecalis*
S4	*S. agona*
S5	*S. simulans*
S6	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona*
S7	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. simulans*
S8	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona*, *S. simulans*
S9	*Ps. aeruginosa*, *E. coli*, *S. agona*
S10	*Ps. aeruginosa*, *E. coli*, *S. agona*, *S. simulans*
S11	*Ps. aeruginosa*, *E. coli*, *S. simulans*
S12	*Ps. aeruginosa*, *E. faecalis*, *S. agona*
S13	*Ps. aeruginosa*, *E. faecalis*, *S. simulans*
S14	*Ps. aeruginosa*, *E. faecalis*, *S. agona*, *S. simulans*
S15	*Ps. aeruginosa*, *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. aeruginosa*, *E. coli*
S22	*Ps. aeruginosa*, *E. faecalis*
S23	*Ps. aeruginosa*, *S. agona*
S24	*Ps. aeruginosa*, *S. simulans*
S25	*Ps. aeruginosa*, *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*

cultures were grown to approximately 10^8 cfu/mL, and the cell concentration was determined by plate counting. Controlled mixed-microbial populations (Table 3) were made by transferring 1 mL of the overnight pure broth culture into a sterile 10 mL bottle and filling with fresh MHB to 10 mL. This was to ensure that each sample had an equal number of cells.

Another set of controlled mixed-microbial populations was prepared as above with the addition of 1 mL of the overnight culture of *C. jejuni* (ST-474). The overnight culture of the *C. jejuni* was used as the control in the study as this strain was shown to have the ability to form a good biofilm.

One milliliter of each of the mixed-microbial populations was transferred into a 5 mL of MHB to provide an inoculum. Eight wells of the 96-well polystyrene plates were inoculated with 0.2 mL of the mixed-microbial populations. Each plate also included eight control wells, containing 0.2 mL of uninoculated MHB.

2.4. Biofilm growth

The microtiter plates were covered with a lid and placed inside sealable plastic boxes with microaerobic pouches (Mitsubishi Gas Chemical Company, Ngaio Diagnostic). The boxes were placed on top of a bench shaker and incubated with gentle swirling at 30 rpm in an incubation room at 37 °C for 72 h. New microaerobic pouches were put inside the boxes after 48 h of incubation as recommended by the manufacturer. The biofilm growth conditions were chosen to optimize biofilm formation by *C. jejuni* based on preliminary trials (data not shown).

2.5. Biofilm reading

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 min. After drying, the wells were stained with 0.2 mL of 0.5% of crystal violet and left on the bench for 15 min. 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 min at 42 °C. The stain was released from the biofilm in the wells by adding 0.2 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 microtiter plate reader (ELx808 Ultra Microplate Reader, Bio-tek instruments, Inc.).

To correct for background staining when screening for biofilm formation, the mean optical density obtained for the controls included in each microtiter plate was subtracted from the mean optical density obtained for each of the test strains included in that plate.

2.6. Biofilm formation index

The biofilm formation index (BFI) was used to express biofilm formation by *C. jejuni* in a controlled mixed-microbial population study. This index was used because it measures both the biofilm and the cell growth in the microtiter plate (Niu and Gilbert, 2004; Naves et al., 2008). Different microorganisms have different cell growth therefore by converting the two parameters into an index, comparison of the combinations with and without *C. jejuni* can be analyzed. The biofilm formation index was determined by applying the formula:

$$BFI = (AB - CW) / G \quad (1)$$

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 microorganisms-free medium only and G is the optical density of the cells growth in suspended culture. The guide used to interpret the BFI readings is shown in Table 4.

Table 4
Semi quantitative classification of biofilm formation (Naves et al., 2008).

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

2.7. Cell recovery

To recover cells from the biofilms forming in the wells of the microtiter plates after incubation, the biofilms in the microtiter wells were washed with sterile distilled water and 0.2 mL of Mueller–Hinton broth was added to two of the eight wells. A sterile cotton wool 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. After the detaching cells from the biofilm into the Mueller–Hinton broth, the suspension of cells was serially diluted (10^{-1}–10^{-2}). A volume of 0.1 mL of each dilution was plated on modified cefoperazone charcoal deoxylate agar (mCCDA; Fort Richard, New Zealand) using an automatic spiral plater (Don Whitley Scientific). 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. The non-*campylobacter* bacteria were inhibited in mCCDA (data not shown).

2.8. Statistical analysis

For the biofilm screening of the *C. jejuni* strains, 12 replicates from each strain were collected from each experiment, and the experiments were performed on four separate occasions, while for the mixed-microbial populations studies, six replicates from each sample were collected in each experiment, and the experiments were performed in two separate occasions. Data were analyzed with SAS software, using SAS analysis of variance (Proc ANOVA) with Student t-test or Tukey's test with a critical probability of $P \leq 0.05$.

3. Results

3.1. Biofilm formation by Campylobacter jejuni

The ability of *C. jejuni* strains to form a biofilm was determined using the microtiter plate biofilm assay (Fig. 1). Most of the strains used in this screening had the ability to form a biofilm, with strong biofilm formation observed with one strain of human-derived strains (ST-474) and poultry-derived strains (ST-474, ST-53, and ST-520). These were significantly different to the other isolates tested in their ability to form a biofilm, based on the T-test ($P \leq 0.05$). A poultry isolate, ST-3609 did not form a biofilm under the conditions of the experiment (Fig. 1). ST-45 gave inconsistent results in the study and therefore was not included in further trials (Fig. 1).

3.2. Overall comparison of biofilm formation in controlled mixed-microbial populations with and without Campylobacter jejuni

The strongest biofilm formation combination was observed in the S5 (*Staphylococcus simulans* and *C. jejuni*) combination (Fig. 2). *S. simulans* was a strong biofilm former on its own. The BFI of *S. simulans* was significantly increased with the addition of *C. jejuni* (ST-474) ($P \leq 0.05$). The S29 (*S. simulans* and *Enterococcus faecalis*) combination without *C. jejuni* had the second highest BFI, however, with the addition of *C. jejuni* in the combination, the BFI was significantly reduced ($P \leq 0.05$) even though it still produced a strong biofilm. *Pseudomonas aeruginosa* (S1) and *Salmonella agona* (S4) did not appear to have the ability to form a biofilm on their own in this

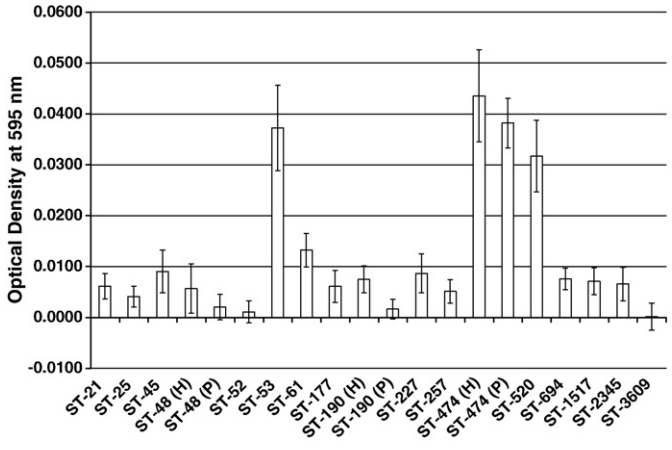

Fig. 1. Biofilm formation on polystyrene microtiter plates by *C. jejuni* strains with gentle swirling (30 rpm) at 37 °C for 72 h under microaerobic conditions. The letter in the bracket represents the source of the strains, 'H' is from clinical human source, and 'P' is from poultry. Trials were performed on four separate occasions, and error bars represent one standard deviation from the mean $P<0.05$ (T-test). ST-53, ST-474 (H), ST-474 (P) and ST-520 formed significantly a stronger biofilm than the other sequence types tested ($P<0.05$).

study, as both of them had a lower BFI when compared with *Escherichia coli* (S2), *E. faecalis* (S3) and *S. simulans* (S5).

3.3. Mixed-microbial populations with Pseudomonas aeruginosa

Most of the combinations that included *Ps. aeruginosa* either failed to form a biofilm or formed a weak biofilm, even though S8 (*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona* and *S. simulans*) without *C. jejuni* formed a moderate biofilm (Fig. 2). There was no significant difference between the S8 combination with and without *C. jejuni* among other low BFI combinations ($P\leq0.05$). The microaerobic atmosphere used in this study may not be suitable for the biofilm formation by *Ps. aeruginosa*. Although the *Ps. aeruginosa*/*C. jejuni* combinations often failed to form biofilm in this study, the turbidity of the wells in the microtiter plate indicated that microbial growth had occurred.

3.4. Mixed-microbial populations with Escherichia coli

E. coli had the ability to form a biofilm in monoculture as well as in certain mixed-microbial combinations (Fig. 2). *E. coli* in this study showed some significant reduction in biofilm formation in mixed-microbial populations, S18 (*E. coli* and *S. simulans*) and S20 (*E. coli*, *E. faecalis*, and *S. simulans*) at $P\leq0.05$. However, there were no differences for most of other *E. coli* combinations with or without *C. jejuni* at $P\leq0.05$.

3.5. Mixed-microbial populations with Enterococcus faecalis

Most of the *E. faecalis* combinations had the ability to form a biofilm except for the S25 *(Ps. aeruginosa*, *E. coli*, and *E. faecalis*) combination without *C. jejuni* (Fig. 2). The S29 (*E. faecalis*, and *S. simulans*) combination had the highest BFI out of all the *E. faecalis* combinations. Strong biofilm formation was observed in S20 (*E. coli*, *E. faecalis*, and *S. simulans*), however, this combination with *C. jejuni* was a moderate biofilm forming combination. *C. jejuni* appeared to reduce the BFI in the S20 combination significantly ($P\leq0.05$).

3.6. Mixed-microbial populations with Salmonella agona

S. agona used in this study did not form a biofilm on its own. S8 (*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona*, and *S. simulans*), S19 (*E. coli*, *E. faecalis*, and *S. agona*) and S27 (*E. coli*, *S. agona*, and *S. simulans*) combinations without *C. jejuni* were moderate biofilm forming combinations, however, with the presence of *C. jejuni*, the biofilm formation was reduced, but not significantly ($P\leq0.05$).

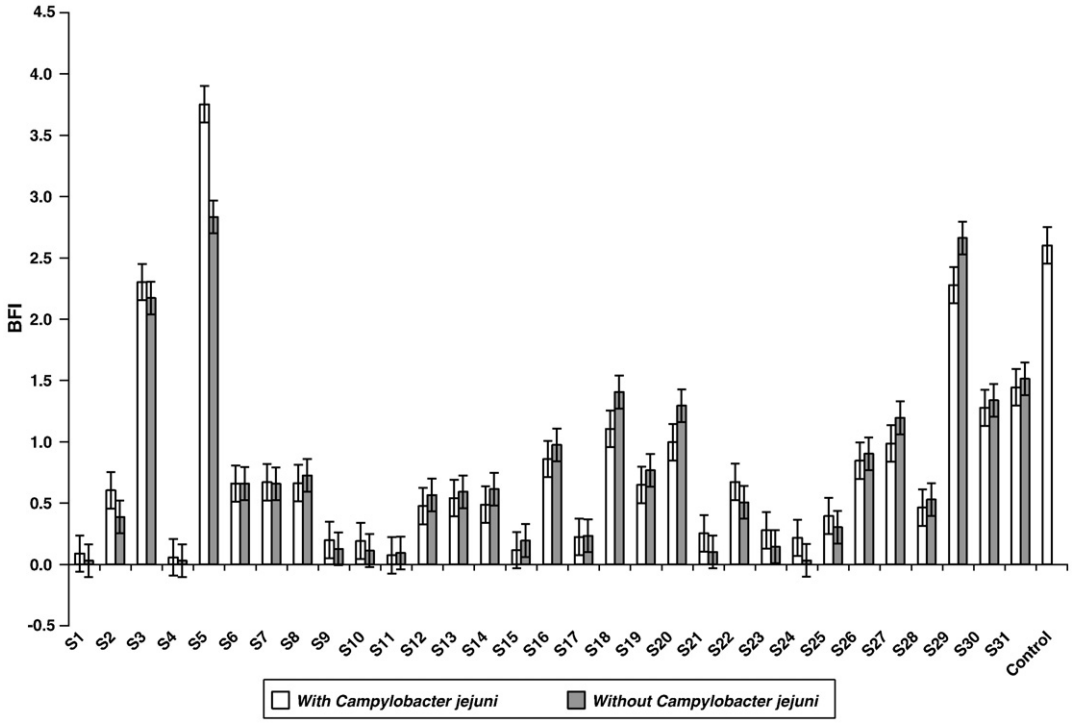

Fig. 2. Biofilm formation on polystyrene microtiter plates by different combinations with gentle swirling (30 rpm) at 37 °C for 72 h under microaerobic conditions. In total, there were 31 combinations with *Campylobacter jejuni*. Trials were performed on two separate occasions with six replicates, and error bars represent standard errors from the mean $P\leq0.05$ (Tukey's test).

3.7. Mixed-microbial populations with Staphylococcus simulans

S. simulans formed a biofilm in monoculture as well as in mixed-microbial population 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*. The S5 (*S. simulans* and *C. jejuni*) combination had the highest BFI when it was grown with *C. jejuni*, followed by *S. simulans* in monoculture (Fig. 2). The BFI for S5 and S29 without *C. jejuni* was similar, however, with the addition of *C. jejuni* into the combinations, the *C. jejuni* enhanced the BFI in S5 while reduced the BFI in S29 significantly ($P \leq 0.05$).

3.8. Comparison of the overall mean of BFI of the controlled mixed-microbial population by specific microorganisms

In general, microorganisms used in this study were able to form a biofilm either alone or in combinations with other microorganisms. Strong biofilm formation was observed in the presence of *S. simulans* followed by *E. faecalis* (Fig. 3). Overall, *C. jejuni* did not show any apparent effect on the biofilm formation by *E. faecalis* or *S. simulans* combinations except in a few controlled mixed-microbial populations.

The other three microorganisms (*Ps. aeruginosa*, *E. coli* and *S. agona*) used in this study were weak biofilm formers, however, mixed-microbial populations that included *Ps. aeruginosa* had the lowest BFI compared with the other microorganisms in this study. There were no differences observed between the biofilm formation of *E. coli* and *S. agona*. *C. jejuni* did not seem to affect the biofilm formation by these microorganisms, as the BFIs with and without *C. jejuni* were similar ($P \leq 0.05$).

3.9. Enumeration and cell recovery of C. jejuni from biofilm

C. jejuni was recovered from biofilms in this study (Table 5). The number of cells recovered ranged from 0 log cfu/mL to 3.42 log cfu/mL. *C. jejuni* was able to attach and survive in most of the combinations, except for most of the *Ps. aeruginosa* combinations.

High numbers of *C. jejuni* were able to be recovered from biofilms formed by *E. faecalis*. Although *S. simulans* was a strong biofilm former, the recovery of *C. jejuni* was not as great as from combinations that included both of the *E. faecalis* and *S. simulans* ($P \leq 0.05$).

4. Discussion and conclusion

The study investigated the growth of *C. jejuni* in specified controlled mixed-microbial populations. Biofilm formation by *C. jejuni* varied from strain to strain. Not all the *C. jejuni* strains tested in the screening of biofilm formation were able to form strong biofilms. Only a few strains were capable of forming a strong biofilm and this agrees with other studies (Joshua et al., 2006; Reeser et al., 2007). Although

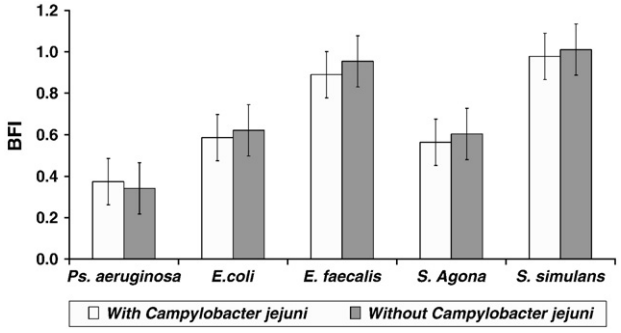

Fig. 3. Comparison of the overall mean of BFI of the controlled mixed-microbial population by specific microorganisms, and error bars represent standard errors from the mean $P \leq 0.05$ (Tukey's test).

Table 5
Enumeration of *C. jejuni* from biofilm.

Sample	Individual/combinations of microorganisms	Log_{10} cfu/mL	Std dev
S1	*Ps. aeruginosa*	0	0
S2	*E. coli*	1.72^{hg}	0.32
S3	*E. faecalis*	3.40^{a}	0.55
S4	*S. agona*	2.04^{efg}	0.50
S5	*S. simulans*	1.81^{fgh}	0.50
S6	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona*	0.50^{ij}	0.54
S7	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. simulans*	0	0
S8	*Ps. aeruginosa*, *E. coli*, *E. faecalis*, *S. agona*, *S. simulans*	0.13^{j}	0.35
S9	*Ps. aeruginosa*, *E. coli*, *S. agona*	0	0
S10	*Ps. aeruginosa*, *E. coli*, *S. agona*, *S. simulans*	0.49^{ij}	0.91
S11	*Ps. aeruginosa*, *E. coli*, *S. simulans*	0.75^{i}	1.04
S12	*Ps. aeruginosa*, *E. faecalis*, *S. agona*	0	0
S13	*Ps. aeruginosa*, *E. faecalis*, *S. simulans*	0	0
S14	*Ps. aeruginosa*, *E. faecalis*, *S. agona*, *S. simulans*	0	0
S15	*Ps. aeruginosa*, *S. agona*, *S. simulans*	0	0
S16	*E. coli*, *E. faecalis*	0	0
S17	*E. coli*, *S. agona*	1.61^{h}	0.39
S18	*E. coli*, *S. simulans*	2.07^{efg}	0.10
S19	*E. coli*, *E. faecalis*, *S. agona*	0.70^{i}	0.59
S20	*E. coli*, *E. faecalis*, *S. simulans*	2.30^{cde}	0.26
S21	*Ps. aeruginosa*, *E. coli*	1.70^{gh}	0.39
S22	*Ps. aeruginosa*, *E. faecalis*	0	0
S23	*Ps. aeruginosa*, *S. agona*	0	0
S24	*Ps. aeruginosa*, *S. simulans*	0	0
S25	*Ps. aeruginosa*, *E. coli*, *E. faecalis*	0.13^{j}	0.35
S26	*E. coli*, *E. faecalis*, *S. agona*, *S. simulans*	1.63^{h}	0.79
S27	*E. coli*, *S. agona*, *S. simulans*	2.14^{def}	0.19
S28	*E. faecalis*, *S. agona*	2.53^{bcd}	0.37
S29	*E. faecalis*, *S. simulans*	3.42^{a}	0.34
S30	*E. faecalis*, *S. agona*, *S. simulans*	2.66^{bc}	0.22
S31	*S. agona*, *S. simulans*	2.78^{b}	0.27

Means with the same letters are not significantly different at $P \leq 0.05$ (T-test).

C. jejuni was able to form biofilms, the amount of biofilm formed was not as great as that formed by other microorganisms used in the study. The ST-474 strains examined, both the poultry and human-derived isolates, did form relatively a strong biofilm, and, if this trait was a consistent finding for this ST, it may help to explain why this sequence type is so prevalent in poultry and in human cases in New Zealand. Extending the study to consider a larger population of STs would be required to test this hypothesis.

In general, *C. jejuni* did not have an effect on biofilm formation in mixed-microbial populations. However, for some specific microbial combinations, there was evidence of an effect of *C. jejuni* on biofilm formation. These effects on biofilm formation may be due to quorum sensing activities or antimicrobial or interspecies competition within the biofilm community (Donlan, 2002).

Strong biofilm forming combinations were observed in the controlled mixed-microbial populations that included either *Enterococcus faecalis* and/or *Staphylococcus simulans*. These two microorganisms were shown to have strong biofilm forming ability when they were grown under microaerobic conditions necessary for *C. jejuni* growth. These microorganisms could provide a safe haven for the *C. jejuni* in the poultry environment, as both of these microorganisms originated from poultry sources and they can be found in both the poultry farm and poultry processing plant environments (Dodd et al., 1988; Georgnaras et al., 1996; Poulsen, 1999). *C. jejuni* has been found to attach on biofilms formed by either *E. faecalis* or *S. simulans* or both (Trachoo and Brooks, 2005; Sanders et al., 2007; Voidarou et al., 2007). Biofilm formation in the poultry environment may reduce if these two microorganisms are reduced or eliminated. 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 formation of biofilm in this study as 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 (Dominguez and Schaffner, 2007; Tuncer and Sireli, 2008). *Ps. aeruginosa* is usually a strong biofilm former, however, in this study; there was no formation of biofilm, which may be due to the growth conditions — in particular the microaerobic conditions needed to support the growth of *C. jejuni*. *Ps. aeruginosa* appeared to have the ability to grow in microaerobic conditions, and is known to survive in anaerobic conditions by pyruvate fermentation (Eschbach et al., 2004). However, *Ps. aeruginosa* did not appear to show any biofilm formation under the conditions used in this study.

The low biofilm formation in *Ps. aeruginosa* mixed-microbial populations may be due to the competitive environment and limited nutrient availability. *Ps. aeruginosa* seemed to inhibit *C. jejuni*, as in most of the mixed-microbial populations that included *Ps. aeruginosa*, as *C. jejuni* was unable to be recovered. *Ps. aeruginosa* has been reported to inhibit *E. coli* and other microorganisms (Gram, 1993; Samelis and Sofos, 2002). 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 (Conner et al., 2005).

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 (Gram, 1993; Samelis and Sofos, 2002; Conner et al., 2005). This effect may not be obvious in the poultry environment, as there are many types of microorganisms that may influence growth and biofilm formation. However, this study has shown that *Ps. aeruginosa* may inhibit or reduce biofilm formation by five other types of microorganisms associated with the poultry environment, in a mixed-microbial setting under microaerobic conditions.

C. jejuni was able to be recovered from biofilms of most of the mixed-microbial populations in this study. *C. jejuni* has the ability to attach and survive in the biofilms, even though the number of cells recovered varied between the different mixed-microbial populations (Trachoo and Frank, 2002; Trachoo et al., 2002; Sanders et al., 2007; Hanning et al., 2008). *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 in this study. Both *E. faecalis* and *S. simulans* were strong biofilm formers 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 therefore be a useful indicator of *C. jejuni* contamination (Awan and Matsumoto, 1998; Pleydell et al., 2010).

The presence of *C. jejuni* in biofilms can be a problem to the poultry industry, as biofilms can protect *C. jejuni* from environmental physical and chemical stress, with the persistent biofilm providing a source of contamination of poultry meat (Denyer et al., 1993; O'Toole et al., 2000; Donlan, 2002; Hall-Stoodley et al., 2004). In this study, *C. jejuni* survived in a wide range of specified mixed-microbial populations, however, most of the mixed-microbial populations that included *Ps. aeruginosa* were not able to harbour *C. jejuni*.

The inhibitory effect of *Ps. aeruginosa* would be interesting to explore further and determine the mechanism of inhibition and the potential of using this as a natural control measure to control *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* was shown to have the ability to attach and survive in certain mixed-microbial populations in this study. However this does not represent the overall microbial population in nature, as only a few of the microorganisms types were used in the study. Although this could not represent the whole microbial population, it is a good indication of microorganism types that may promote biofilm formation in the poultry industry.

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 under different growth conditions and surfaces. The goal should be to establish control measures that limit the formation of biofilms containing *C. jejuni* in poultry farms and poultry processing plants to reduce the reservoir of contamination and thus reduce the incidence of campylobacteriosis. The inhibitory effect of *Ps. aeruginosa* needs to be identified with the view to making use of this effect in controlling biofilm development.

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