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Attachment and Heat Resistance of *Campylobacter jejuni* on *Enterococcus faecium* Biofilm

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Abstract: Attachment and heat resistance of *Campylobacter jejuni* in *Enterococcus faecium* biofilm were studied. *E. faecium* biofilm were incubated with 10^7 cfu mL^{-1} *C. jejuni* for 4.5 h at 23°C under atmospheric conditions or 12 h at 42°C under microaerophilic conditions. The coupons were heat treated at 56 and 63°C for 30, 60 and 120 sec. In addition, *C. jejuni* in *E. faecium* biofilm was incubated for up to 4 days at 23°C under atmospheric conditions in 50% trypticase soy broth for survival study. Hydrophobicity of *C. jejuni* planktonic cells, *E. faecium* planktonic and biofilm cells, as determined by salt aggregation test and microbial adhesion to hydrocarbon test using hexadecane were determined. D-value of *C. jejuni* planktonic cells at 56 and 63°C were 46 and 12 sec, respectively. D-value of *E. faecium* planktonic cells at 56 and 63°C were 117 and 80 sec, respectively. The presence of *E. faecium* biofilm reduced the lethal effect of heat on *C. jejuni* cells when heated at 56 and 63°C. *C. jejuni* formed biofilm on stainless steel when grown at 42°C under microaerophilic conditions for 12 h but the biofilm did not survive the heat treatments nor did *C. jejuni* cells in *E. faecium* biofilm. *C. jejuni* in biofilm persisted under atmospheric condition at 23°C for up to 2 days while *C. jejuni* attached on stainless steel without biofilm could not be recovered after two days of incubation. After forming *E. faecium* biofilm, became more hydrophobic than its planktonic cells. This may attract *C. jejuni* cells to attach on the biofilm as *C. jejuni* was found to be more hydrophobic than *E. faecium* planktonic cells.

Key words: *C. jejuni*, *E. faecium*, biofilm, attachment

INTRODUCTION

Campylobacter jejuni is the most commonly reported cause of bacterial diarrhea worldwide. The cost of *Campylobacter* infections in New Zealand during 1994 was estimated as $NZ 61.7 million[1]. In 1995, estimated cost of the impacts was 4.48 million[2]. McNicholus *et al.*[3] concluded that the emergence of *Campylobacter jejuni* infections was not due to changes in laboratory methodologies over the last 5 years. As the current high incidence of *Campylobacter* infection has considerable financial impact on the community, further public health and research initiatives to decrease incidence and associated costs should be encouraged.

Despite its susceptibility to atmospheric oxygen and inability to grow at ambient temperature, *C. jejuni* has been detected in natural aquatic environments including river water, ground water, coastal water and lake water[4-7]. A survey on *C. jejuni* in river, drinking, roof and shallow ground waters of New Zealand indicated that water may be an important source of *C. jejuni*[8]. Biofilm is highly prevalent in aquatic environments which are found in the nature and food processing facilities. Studies have shown that biofilms improved the survival of *C. jejuni* in aquatic environment[9,10] and biofilms improved *C. jejuni* survival during chemical sanitizer treatment[11].

A recent study on *C. jejuni* attachment to mucus membrane in canine's gut[12] showed that two strains of *Enterococcus faecium* enhanced the attachment of *C. jejuni* from 134 to 205% when the initial inoculum level was 10^7-10^8 cfu mL^{-1}. *E. faecium* is commonly found in farms, food processing plants and even in the gut of some animals. It is fed to animals as probiotic culture to promote healthy living. *E. faecium* was shown to readily attach to stainless steel surface and form biofilm within 2-4 h depending on initial inoculum level[13]. The effect of preformed *E. faecium* biofilm on *C. jejuni* attachment and survival is therefore of interest. The objective of this study was to investigate the effect of preformed *E. faecium* biofilm on stainless steel on the attachment, survival and heat resistance of *C. jejuni*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions: Reference culture of *C. jejuni* ATCC 29428 was obtained from New Zealand Culture Collection (Wellington, New Zealand). The culture was then activated three times on semisolid Brucella-reducing medium at 42°C in microaerobic environment using Oxoid CampyGen (Hampshire, England). Brucella-reducing medium contains Brucella broth with 1.5 g L^{-1} agar and ferrous bisulfate pyruvic stock solution added to provide 0.5 g L^{-1} ferrous sulfate; 0.2 g L^{-1} sodium bisulfate and 0.5 g L^{-1} pyruvic acid (Sigma Chemical Co., St. Louis, Mo). The stock solution was stored at -80°C. *E. faecium* from Massey University's culture collection was previously isolated from a dairy plant and was activated three times in Trypticase soy broth (TSB, BBL Microbiology Systems, Cockeysville, Md) at 30°C for 18 h before use. Stock cultures were stored in TSB with 20% glycerol at -80°C.

Hydrophobicity and aggregation: Relative hydrophobicity of *C. jejuni* and *E. faecium* were determined by the bacterial adhesion to hydrocarbon (BATH) method[14]. Briefly, *C. jejuni* and *E. faecium* were grown on Brucella-reducing agar at 42°C for 48 h and TSB at 30°C for 18 h, respectively. *E. faecium* biofilm cells were removed from stainless steel surface by vortexing with sands[15]. Cell suspension of each strain with optical density (OD_{600}) at 600 nm between 0.8-1.0 was prepared in sterile deionized water or Phosphate Buffer Saline (PBS). Equal volume of hexadecane was added and mixed to cell suspension by vortexing (Vortex Genie 2; Fisher Scientific Co.) at the highest setting for 30 sec. The mixture of hydrocarbon and cell suspension was incubated at 25°C for 20 min to allow complete phase separation. Relative hydrophobicity was determined by percent changes in OD_{600} of cell suspension after the addition of hydrocarbon. Hydrophobicity was also determined using salt aggregation test (SAT)[16]. Each strain was suspended in sterile PBS (pH 7.2) to a final OD_{600} between 0.8-1.0. The cell suspension was added with an equal volume of 0.2, 0.5, 1, 2 or 3 M of ammonium sulfate and mixed on an orbital shaker for 2 min. Each well was determined for microscopic aggregation compared to the control without ammonium sulfate (PBS and culture) using a stereo microscope (SZ51, Olympus, Japan). Strains that aggregate with less than 1 M ammonium sulfate were considered hydrophobic.

Autoaggregation was determined using the method described by Misawa and Blaser[16]. Briefly, cell suspensions with OD_{600} of 1 were prepared similarly to those for MATH test. To determine % coaggregation (two strains), equal volume (1.5 mL) of *E. faecium* biofilm cell suspension and *C. jejuni* cell suspension were mixed by vortexing and Initial OD_{600} was measured. After the 24 h incubation period at 20°C, OD_{600} was measured again. Percent change in OD_{600} was considered %autoaggregation.

Determination of decimal value: D-values at 56 and 63°C of *C. jejuni* and *E. faecium* planktonic cells were determined by a test tube method. Thin-walled test tubes, 180×9 mm ID, containing 9 mL of 0.1% peptone, were pre-heated in a water bath at 56°C or 63°C. Bacterial suspensions, 1 mL, were added to the tubes. After 30 sec, 1, 3, 5, 8, 10 and 20 min, triplicate 0.1 mL volumes were plated on Brucella-reducing agar with 50 mg L^{-1} 2,3,5-triphenyl tetrazolium chloride (TTC)[10] for *C. jejuni*. Plates were incubated at 42°C for 48 h in a microaerobic environment for *C. jejuni*. *E. faecium* was enumerated by measuring impedance signal using a microbial analyzer, BacTrac 4100 (Sy-Lab VgmbH, Vienna, Austria). A calibration curve was determined by plotting a graph of log population of *E. faecium* enumerated on TSA plate at 30°C for 18 h under aerobic environment against impedance signal. The instrument was set to record the impedance signal every 10 min interval for 20 h. The equation (r = 0.9836) obtained from the calibration curve was

$$\log CFU = -0.4413*t + 8.7174$$

"t" is time in h required to obtain the threshold (7% M and E value)

Survival and heat resistance of *C. jejuni* in *E. faecium* biofilm: *E. faecium* was grown in 50% TSB (HTSB) on 1×1×0.1 cm stainless steel coupons (Type 316, 2B surface finish) for 3 days at 30°C. Media was refreshed every 24 h. This resulted in about 3 log cells cm^{-2} of *E. faecium* biofilm cells. Preformed *E. faecium* biofilm was incubated with 10^7 cfu mL^{-1} *C. jejuni* suspension for 18 h to allow attachment. Stainless steel coupons were then transferred into new HTSB media. This was considered day zero. Incubation at 23°C under atmospheric condition continued for 4 more days. Media was refreshed every 24 h and coupons were gently rinsed with sterile water to eliminate loosely attached cells. Control without biofilm was included. *C. jejuni* suspension was prepared by inoculating *C. jejuni* in Brucella-reducing broth and incubating at 42°C for 48 h under microaerobic environment.

Heat resistance of *C. jejuni* in *E. faecium* biofilm was determined by heating the inoculated coupons at 56 or 63°C for 30, 60 and 120 sec. To study the effect of attachment parameters on heat resistance of *C. jejuni* in *E. faecium* biofilm, *C. jejuni* was allowed to attach to *E. faecium* biofilm at 23°C under atmospheric condition for 4.5 h or at 42°C under microaerobic condition for 12 h. Samples giving log cells cm^{-2} less than the detection limit of 1.6 log cells cm^{-2} were enriched in Brucella-reducing broth and incubated at 42°C for 72 h under microaerobic environment to confirm the inactivation of *C. jejuni*.

Data analysis: Samples were stained with Acridine Orange (0.1 g L^{-1} in 0.5 M acetate buffer: pH 3.5-4.0) for 1 min and rinsed with deionized water. The coupons were then viewed under an epiflourescence microscope(Nikon Optiphot 2 equipped with a Nikon digital camera Collpix E995, Tokyo, Japan) using a 405-nm excitation filter and 525-nm emission filter. The same frozen stock cultures and equipment were used in all replicates. Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA and GLM. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined by least square means at $p = 0.05$.

RESULTS AND DISCUSSION

Attachment of *C. jejuni* to *E. faecium* biofilm: Hydrophobicity of *E. faecium* was less than that of the planktonic culture (Table 1). This was confirmed by the two methods used to determine relative hydrophobicity in this study. It has been reported that bacteria at different growth modes differ in their hydrophobicity. Spores of *Bacillus* and *Clostridium* were more hydrophobic than their vegetative cells[17]. *C. jejuni* grown in biofilm mode were also more hydrophobic than those grown in planktonic mode[18]. Attached cells thus seem to possess hydrophobicity characteristics. *C. jejuni* relative hydrophobicity was 3.90%, lower than those of *E. faecium* (8.85%) and *E. faecium* biofilm (24.09%). *E. faecium* with higher hydrophobicity readily forms biofilm on stainless steel and may attract *C. jejuni* to attach to the *E. faecium* biofilm. Aggregation of microorganisms enhances biofilm formation and mediates attachment of pathogen on biofilms[19]. Autoaggregation of *E. faecium* was significantly higher than that of *C. jejuni* (Table 1). This implies that *E. faecium* had more ability to initiate biofilm formation when compared to *C. jejuni*. Although initial attachment of *C. jejuni* in this study was high enough to subsequently form biofilm, *C. jejuni* don't grow at 23°C under atmospheric oxygen tension[10]. The attachment to surfaces by microorganisms and subsequent formation of biofilm is ubiquitous[20] and requires an effective cleaning program to control[21]. *E. faecium* are commonly found in food processing plants, grow in a wide range of temperature from 10 to 45°C[22] and readly form biofilm on stainless steel[13]. If both *E. faecium* and *C. jejuni* are present in an aqueous system, it is likely that *E. faecium* will quickly form biofilm and integration of *C. jejuni* into the biofilm can subsequently occur. Figure 5 shows the attachment of *C. jejuni* on 18 h *E. faecium* biofilm and some *C. jejuni* cells on the stainless steel surface.

It is conceivable that *C. jejuni* preferably attaches to *E. faecium* biofilm. This may be due to the compatibility of their surface physicochemical properties such as hydrophobicity and autoaggregation. *C. jejuni* is a major foodborne pathogen causing bacterial diarrhea via contaminated foods, raw milk and water[23-25] in may countries. *E. faecium* biofilm can become a potential source of pathogenic *C. jejuni* contamination in food processing plants, especially in poultry processing plants where the animals are natural reservoirs of *C. jejuni*.

Heat resistance of *C. jejuni* in *E. faecium* biofilm: The previously reported D-values of *C. jejuni* varied depending on culture strain, heating medium and methods. In this experiment, we used pre-heated 0.1% peptone in thin-wall test tube to determine the D-value of *C. jejuni* (46 sec). In a previous study[26], Blankenship and Craven used the same method and heating medium and reported the *C. jejuni* D55°C of 38 sec slightly less than our result. Other report indicates that *C. jejuni* is fairly susceptible to heat[27]. D-values of *E. faecium* and *C. jejuni* were significantly different (Table 2). *E. faecium* was more resistant to heat at 56 and 63°C than *C. jejuni*. This may explain the high prevalence of *E. faecium* in food processing plants.

Survival of *C. jejuni* decreased with time (Fig. 1). After 2 days of incubation in atmospheric condition, the number of *C. jejuni* without biofilm were undetectable by culturing method while the viability of *C. jejuni* within the

Table 1: Hydrophobicity as determined by microbial adhesion to hydrocarbon (hexadecane) and salting aggregation test (ammonium sulfate) and aggregation of *C. jejuni*, *E. faecium* and *E. faecium* biofilm[1]

Microorganism	Hydrophobicity (%)	Hydrophobicity[2] (Ammonium sulfate)	Autoaggregation (%)
C. jejuni	3.90[c][3]	++	45.6[b]
E. faecium	8.85[b]	+	72.5[a]
E. faecium biofilm	24.09[a]	+++	Not tested

[1]Means of four replications

[2]Ammonium sulfate concentrations were 0.2-4 M; high concentration of ammonium sulfate used to aggregate bacterial suspension in PBS (pH 7.2) indicates high degree of hydrophobicity on cell surface

[3]Means in column with no common letter differ at $p<0.05$

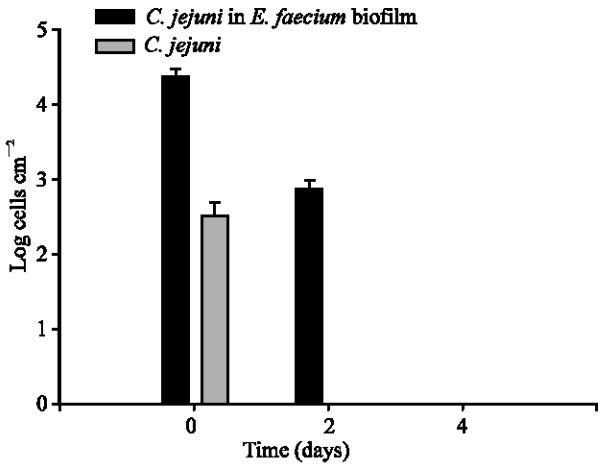

Fig. 1: Survival of *C. jejuni* in biofilm of *E. faecium* (black bars) and *C. jejuni* without biofilm (grey bars) on stainless steel after 0.2 and 4 days of incubation at 23°C under aerobic condition in 50% TSB *E. faecium* biofilm (3 log cells cm^2) was incubated with 10^7 cfu mL^{-1} *C. jejuni* for 18 h to allow attachment at 23°C under aerobic coindition

Table 2: D-value[1] of *C. jejuni* and *E. faecium* planktonic cells at 56°C and 63°C

Microorganism	$D_{56°C}$ (sec)±SD	$D_{63°C}$ (sec) ±SD
C. jejuni	46.45±1.50	12.22±1.05
E. faecium[2]	116.78±2.34	80.40±3.21

[1]Means of three replications
[2]Heat treated *E. faecium* was enumerated by measuring impedance signal

biofilm of *E. faecium* was extended. However after 4 days of incubation, neither *C. jejuni* with nor without biofilm could not be detected by culturing method. This showed that *E. faecium* biofilm enhanced the survival of *C. jejuni* under atmospheric conditions. Other authors also found that biofilm from various sources improved survival of *C. jejuni*[9,10]. Trachoo *et al.*[10] reported that biofilm formers isolated from a chicken house improved survival of *C. jejuni* (chicken isolate). Since *C. jejuni* is sensitive to atmospheric oxygen, reduced oxygen concentration within biofilm[28] may improve *C. jejuni* survival. The presence of *E. faecium* biofilm increased attachment of *C. jejuni* on stainless steel by 1-2 log cell cm^{-2} (Fig. 1 and 3). This also confirms the finding of Rinkinen *et al.*[12] who reported that the probiotic *E. faecium* increased the attachment of *C. jejuni* in canine's gut model. In the present study, *E. faecium* was enumerated by impedance method while *C. jejuni* was enumerated by plate count method. Although impedance method generally recovered more cells than plate count method[29], it was not possible to incubate *C. jejuni* sample in the BacTrac system under microaerobic condition. Heat resistance of *C. jejuni* in biofilm at 56°C of *E. faecium* was studied when attachment occurred under atmospheric

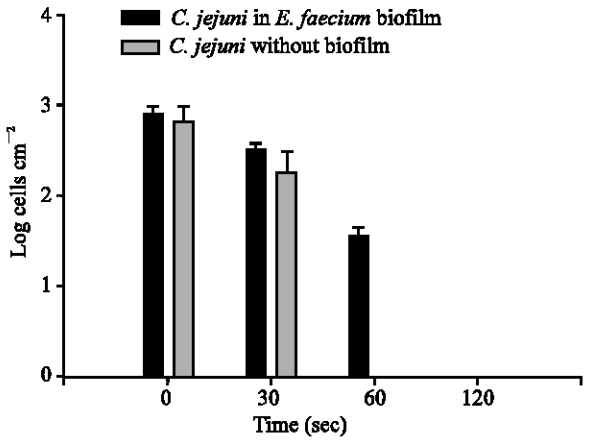

Fig. 2: Survival of *C. jejuni* in biofilm of *E. faecium* and *C. jejuni* without biofilm after heat treatment at 56°C for 30, 60 and 120 sec. *C. jejuni* (10^7 cfu mL^{-1}) was incubated with stainless steel coupons with preformed biofilm of *E. faecium* (3 log cells cm^2) and without biofilm at 42°C under microaerobic condition for 12 h

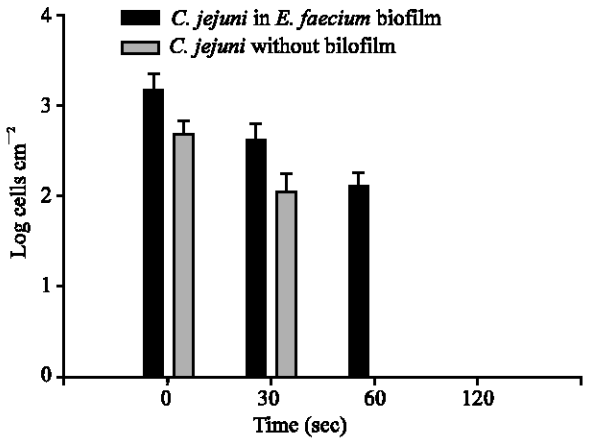

Fig. 3: Survival of *C. jejuni* in biofilm of *E. faecium* and *C. jejuni* without biofilm after heat treatment at 56°C for 30, 60 and 120 sec. *C. jejuni* (10^7 cfu mL^{-1}) was incubated with stainless steel coupons with preformed biofilm of *E. faecium* (3 log cells cm^2) and without biofilm at 23°C under microaerobic condition for 4.5 h

(23°C) and microaerobic environment (42°C). The numbers of *C. jejuni* attached to stainless steel with and without biofilm of *E. faecium* at 42°C under microaerobic environment were similar (Fig. 2). In contrast, more *C. jejuni* attached to stainless steel at 23°C under atmospheric environment with preformed *E. faecium* biofilm compared to clean stainless steel (Fig. 3). Optimal

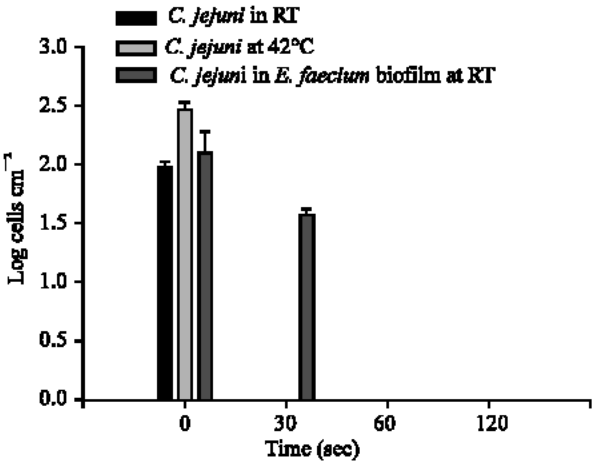

Fig. 4: Survival of *C. jejuni* in biofilm of *E. faecium* and *C. jejuni* without biofilm after heat treatment at 56°C for 30, 60 and 120 sec. *C. jejuni* (10^7 cfu mL^{-1}) was incubated with stainless steel coupons with preformed biofilm of *E. faecium* (3 log cells cm^2) and without biofilm at 42°C under microaerobic condition for 12 h or room temperature under atmospheric condition for 4.5 h

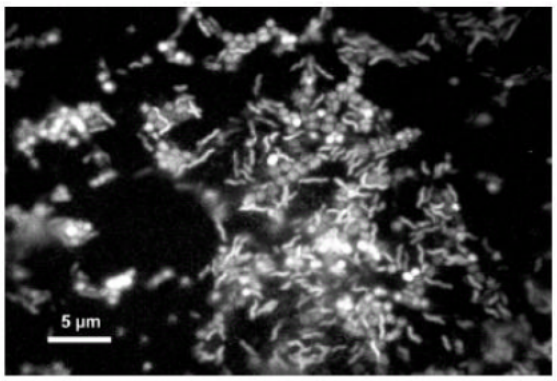

Fig. 5: Attachment of *Campylobacter jejuni* (spiral) on 18 h *Enterococcus faecium* biofilm (cocci) after 2 h

growth conditions for *C. jejuni* were at 42°C and under microaerobic environment[30]. Since *C. jejuni* does not grow at 23°C, the bacteria ability to attach to surfaces may be reduced. Motility of the viable *C. jejuni* may also play important role on attachment.

Present results showed that *C. jejuni* without biofilm did not survive heat of 56°C more than 60 sec and heat treatment at 56°C for 120 sec inactivated both *C. jejuni* with biofilm and without biofilm. Effect of biofilm on heat resistance was reduced (Fig. 2) when *C. jejuni* was attached to stainless steel surface at 42°C under microaerobic conditions when compared with attachment at 23°C under atmospheric condition indicating metabolic activity of *C. jejuni* during attachment was crucial for acquiring the improved survival rate. *C. jejuni* attached and formed biofilm under the optimal growth conditions, 42°C and microaerobic conditions (Fig. 4). Under such conditions, more *C. jejuni* attached (2.4 log cells cm^{-2}) to clean stainless steel surface compared to attachment at 23°C (room temperature) with (2.1 log cells cm^{-2}) or without biofilm (1.9 log cells cm^{-2}). Although more attached to the stainless steel under the optimal growth conditions, *C. jejuni* did not survive heat treatment at 63°C for 30 sec. After high heat treatment at 63°C for 60 sec, *C. jejuni* with and without biofilm of *E. faecium* were completely inactivated regardless of attachment conditions. This indicates that *C. jejuni* is a heat sensitive pathogen. However in the presence of biofilm, *C. jejuni* heat resistance was increased. Not only does biofilm provide protection from heat treatment, but also protection from chemical sanitizers. Trachoo and Frank[11] reported that survival of *C. jejuni* after the treatment of selected chemical sanitizers (50 and 200 ppm of chlorine, quaternary ammonium, peracetic acid and peroxyacetic acid) was enhanced by the presence of biofilm. *E. faecium* survived at 70°C[31] and resisted selected chemicals in the group of aldehydes, phenols, quaternaries and oxidizing agents[32]. Popular chemical sanitizers like sodium hypochlorite and quaternary ammonium compounds were reported to be not very effective against *E. faecium* biofilm on stainless steel[29]. Therefore it is believable that *E. faecium* biofilm can provide protection from chemical sanitizers to *C. jejuni*.

E. faecium are commonly found in food processing plants and grow in a wide range of temperature from 10 to 45°C[22] and readily form biofilm[13]. In the present study, *E. faecium* biofilm increased the attachment of *C. jejuni* to stainless steel surface and also improved *C. jejuni* heat resistance. *C. jejuni* was able form biofilm to under its optimal growth conditions. *E. faecium* biofilm can be potential source of *C. jejuni* in food processing plants due to its prevalence in the nature and its ability to harbor and protect *C. jejuni* from heat treatment and environmental stresses.

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