Inhibition of *Campylobacter jejuni* biofilm formation by D-amino acids

Bassam A. Elgamoudi[1], Taha[1], and Victoria Korolik[1ξ].

[1] Institute for Glycomics, Griffith University, Gold Coast campus, QLD 4222, Australia.

[ξ]Corresponding author, Victoria Korolik, Institute for Glycomics, Griffith University, Gold Coast campus, QLD 4222, Australia, email v.korolik@griffith.edu.au

Abstract: The ability of bacterial pathogens to form biofilms is an important virulence mechanism in relation to its pathogenesis and transmission. Biofilms play a crucial role in survival in unfavourable environmental conditions, act as reservoirs of microbial contamination and antibiotic resistance. For intestinal pathogen *Campylobacter jejuni*, biofilms are considered to be a contributing factor in transmission through the food chain and currently, there are no known methods for intervention. Here we present an unconventional approach to reducing biofilm formation by *C. jejuni* by the application of D-amino acids (DAs), and L-amino acids (LAs). We found that DAs and not LAs, except L-alanine, reduced biofilm formation by up to 70%. The treatment of *C. jejuni* cells with DAs changed the biofilm architecture and reduced the appearance of amyloid-like fibrils. In addition, a mixture of DAs enhanced antimicrobial efficacy of D-Cycloserine (DCS) up to 32% as compared with DCS treatment alone. Unexpectedly, D-alanine was able to reverse the inhibitory effect of other DAs as well as that of DCS. Furthermore, L-alanine and D-tryptophan decreased transcript levels of peptidoglycan biosynthesis enzymes alanine racemase (*alr*) and D-alanine-D-alanine ligase (*ddlA*) while D-serine was only able to decrease the transcript levels of *alr*. Our findings suggest that a combination of DAs could reduce biofilm formation, viability and persistence of *C. jejuni* through dysregulation of *alr* and *ddlA*.

Keywords: D-amino acids, *Campylobacter jejuni*, Biofilm, Alanine racemase, CLSM, confocal laser scanning microscopy

Introduction

Human pathogen *Campylobacter jejuni* is a leading foodborne bacterial cause of diarrhoeal disease which, according to the World Health Organization (WHO), occurs annually in approximately 10% of the world's population, including 200 million children (1, 2). Campylobacters are increasingly resistant to antibiotics which is enhanced by their ability to form biofilms (3-5). *C. jejuni*, in particular, is able to form mono- and mixed-culture biofilms *in vitro* and *in vivo* (6), which recognized as a contributing factor of *C. jejuni* transmission through the food chain where biofilms allow the cells to survive up to twice as long under atmospheric conditions and in water (7-9). Campylobacters exhibit intrinsic resistance to many antimicrobial agents such as cephalosporins, trimethoprim, sulfamethoxazole, rifampicin and vancomycin, and are listed in WHO list of priority pathogens for new antibiotics development (3, 4, 10-15). Biofilms are known to enhance antimicrobial resistance of many pathogens (3-5, 16); thus, unconventional approaches to controlling biofilms and improving the efficacy of currently used antibiotics are urgently needed. Recent investigations into potential antimicrobials include naturally occurring small molecules such as nitric oxide, fatty acids, and D-amino acids (DAs) (17-20). DAs showed an ability to disperse some bacterial biofilms *in vitro*, such as those formed by *Bacillus subtilis*, *Staphylococcus aureus* , *Enterococcus faecalis* and *Pseudomonas aeruginosa* (21-26). It is well documented that microorganisms preferentially utilize L-amino acids (LAs) over DAs

(27, 28), yet naturally occurring DAs have been found in different environments, such as soil, as well as in human and animals tissues (27). In addition, many bacterial species secrete DAs in the stationary growth phase and when encased in biofilms. For example, *Vibrio cholerae* can produce D-methionine (D-met) and D-leucine (D-leu), while *B. subtilis* generates D-tyrosine (D-tyr) and D-phenylalanine (D-phe) which can accumulate at millimolar concentrations (29, 30). The ability of bacteria to produce DAs is proposed to be a mechanism for self-dispersal of aging biofilms, and DA production may also inhibit the growth of other bacteria during maturation of mixed biofilms. In a naturally occurring biofilms, DAs are found to be involved in the regulation of extracellular polymeric saccharide (EPS) production, for instance, D-tyr reduces the attachment of *B. subtilis*, *S. aureus* and *P. aeruginosa* to surfaces (22, 24, 31-33). Also, DAs can induce disassembly of matrix-associated amyloid fibrils that link the cells within the biofilm and contribute to the biofilm strength (34). Effective concentration of DAs required to inhibit the biofilm formation varies depending on bacterial strain and DAs concentration ranging between 3 μM and 10 mM (23, 33, 35, 36). It's important to note that some DAs exhibit inhibitory or toxic effects on a number of bacterial species and can interfere with the activities of peptidases and proteases involved in cell wall synthesis, for example, D-met can be incorporated into the peptidoglycan (PG) of bacterial cell walls, causing morphological and structural damage (37).

DAs appear to be able to disrupt the biofilms via multiple mechanisms, offering an advantage to other biofilm dispersal agents which target a single process essential for biofilm formation, indicating that DAs could form basis for a potential antibiofilm agent.

Herein, we demonstrate that D-alanine (D-ala), L-alanine (L-ala), D-serine (D-ser), D-methionine (D-met), and D-tryptophan (D-trp) can inhibit and disperse biofilms formed by *C. jejuni* and *C. coli* and that it may be possible to use these DAs to enhance the efficacy of antibiotics such D-cycloserine. Also, we present evidence that DAs target alanine racemase (*alr*) in *C. jejuni,* which leads to the inhibition of growth and biofilm formation. This finding may be the key to understanding the mechanisms of DAs action and also could provide an alternative strategy to control *Campylobacter* spp transmission via the food chain.

Results

Effect of LAs and DAs on biofilm formation by *C. jejuni*

In order to investigate the effect of LAs and DAs on biofilm formation, different concentrations of LAs and DAs (0.1-100 mM) were tested for their ability to disrupt or disperse the *Campylobacter* biofilm. Two assays were applied, one to measure the percentage of biofilm inhibition (%) (Inhibition Assay) and the other to determine the effect on the dispersion of a formed biofilm (Dispersion Assay). Treatment of *C. jejuni* culture with DAs showed significant inhibitory effect ($P < 0.001$) on biofilm formation. Prescreening of individual LAs and DAs identified four (D-ala, D-met, D-ser, and D-trp) that had a potent ability to inhibit biofilm formation by *C. jejuni* (Fig 1). In contrast, the L-form of those amino acids, except L-ala, had no inhibitory effect, and some of them, L-met and L-trp, significantly increased biofilm formation.

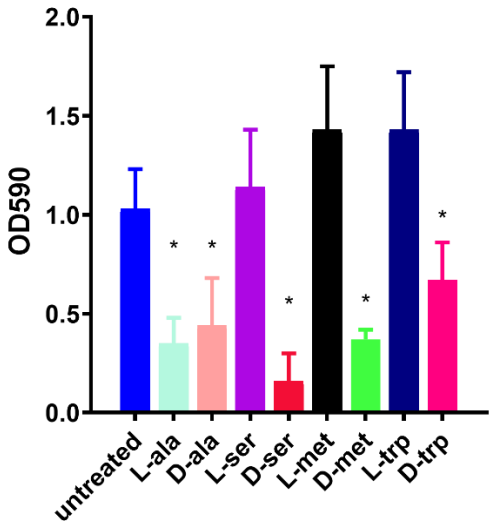


Fig 1. Effect of 100 mM DAs and LAs on *C. jejuni* 11168-O biofilm. Inhibition of biofilm formation in the presence of 100 mM of; L-alanine (L-ala), D- alanine (D-ala), L-serine (L-ser), D-serine (D-ser), L-methionine (L-met), D-methionine (D-met), L-tryptophan (L-trp), or D-tryptophan (D-trp). The asterisk (*) indicates a statistically significant difference using the unpaired Student's t-test, $p<0.05$.

The DAs had a strong inhibitory effect on biofilm formation by *C. jejuni* at 10 mM concentration, with 48% inhibition for D-trp, while D-ala reduced biofilm formation by 28%. Interestingly, 50 mM L-ala reduced biofilm by up to 63% as compared to 45% by D-ala at same concertation (Fig 2). DAs had a disruptive effect on the existing biofilm where D-ser had the most significant effect ($P < 0.001$) on formed biofilm disruption, up to 71%, at 50 mM (Fig 2), and the addition of 10 mM D-trp led to 42% disruption of formed biofilm. Based on the results of DAs inhibitory and dispersal activities, the concentration between 5 to 10 mM was chosen for all subsequent assays.

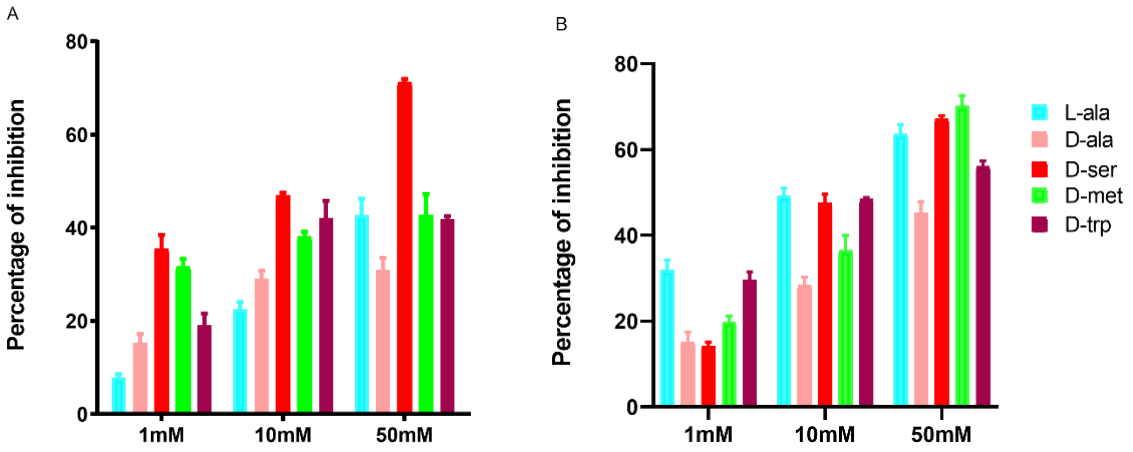


Fig 2. Inhibition and dispersion response of *C. jejuni* 11168-O biofilms in the presence of LAs and DAs at different concentrations. A) Dispersion of the existing biofilm induced by different concentrations of LAs and DAs. B) Inhibition of biofilm formation by different concentrations of LAs and DAs.

In order to elucidate strain-specific responses, *C. jejuni* 11168-O, *C. jejuni* 81-176, and *C. coli* NCTC 11366, were used to confirm the inhibitory effect of D-ala, D-ser, D-met, and D-trp at 10 mM. The effect of DAs on biofilm formation was strain-dependent, where D-ser and D-trp had the greatest inhibitory effect on biofilm formation by 11168-O, D-ala and D-met were most effective against 81-176, and *C. coli* (Fig 3).

The equimolar mixture of DAs and LAs (1:1) showed ≥ 40% inhibition of *C. jejuni* 11168-O biofilm formation (Fig 4). The mixture of the four amino acids, L-ala, D-met, D-ser, D-trp (5:5:2:5 mM), was more potent, with up to 49% inhibition of biofilm formation; however, the addition of D-ala to D-ser decreased the inhibitory effect (Fig 4).

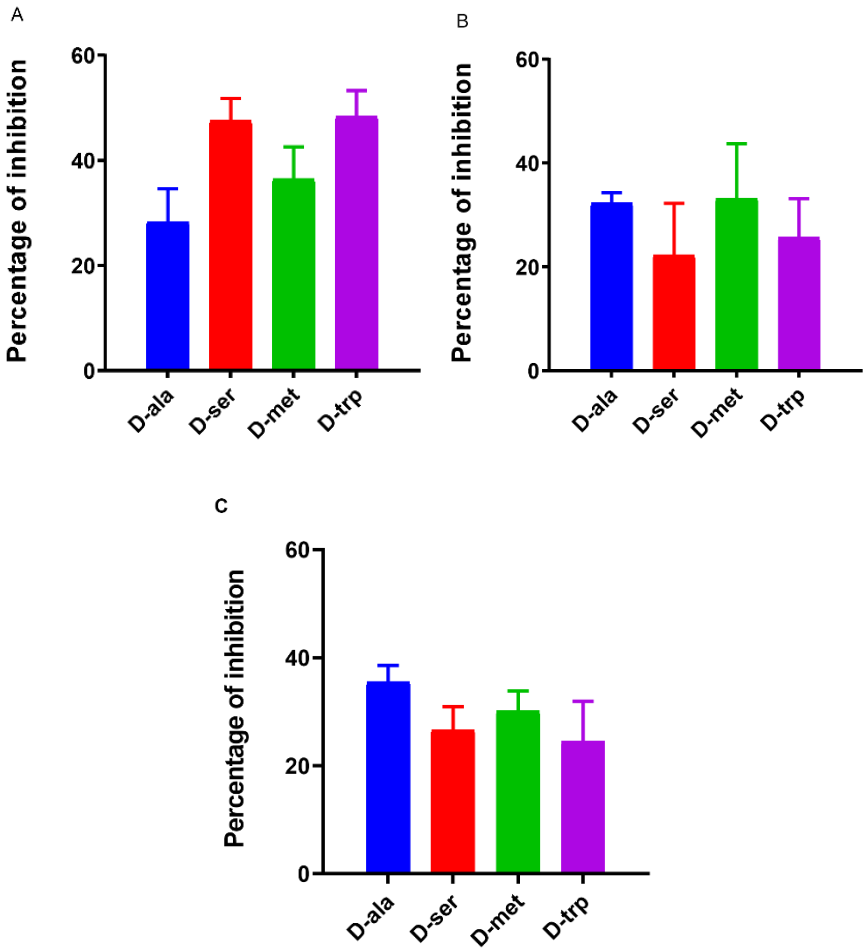


Fig 3. Quantitative analysis of biofilm inhibition of A) *C. jejuni* 11168-O, B*) C. jejuni* 81-176, and C) *C. coli* NCTC 11366 in the presence of 10 mM of DAs.

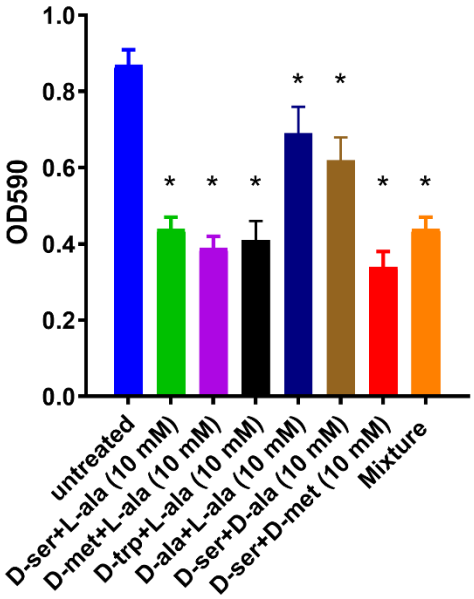


Fig 4. Effect of the equimolar mixture of DAs and LAs on *C. jejuni* 11168-O biofilm. The asterisk (*) indicates a statistically significant difference using the unpaired Student's t-test, $p<0.05$.

Microscopic characterization of the dispersion effect of DAs on biofilm formation

Microscopic examination of formed biofilms, treated with individual DAs, by confocal microscopy demonstrated a significant reduction in biofilm formation compared to that of untreated controls (Fig 5). The disassembly of the amyloid fibrils, which connect the cells within the structure of *C. jejuni* biofilms, can also be observed (Fig 6).

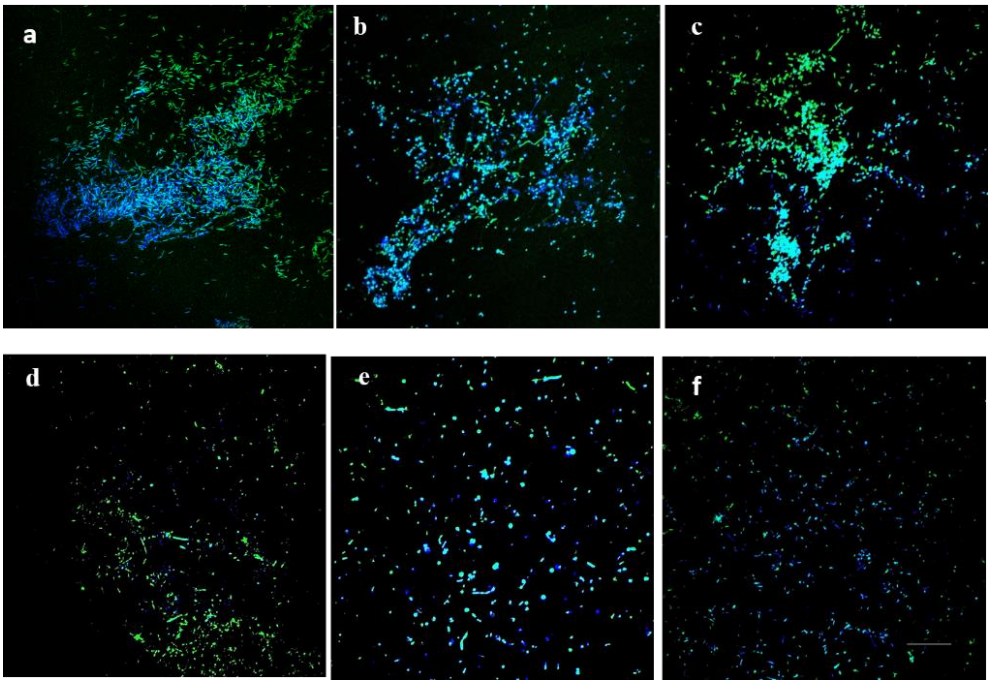


Fig 5. Confocal scanning laser microscopy images of *C. jejuni* 11168-O biofilm in presence of 25 mM of DAs. *C. jejuni* biofilm at 48h, imaged using dual fluorescence labelling by CLSM. a) Untreated, b) D-ala, c) L-ala, d) D-ser, e) D-met, f) D-trp. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) and amyloid fibrils by Thioflavin T (ThioT, green) (Scale bar= 20µm).

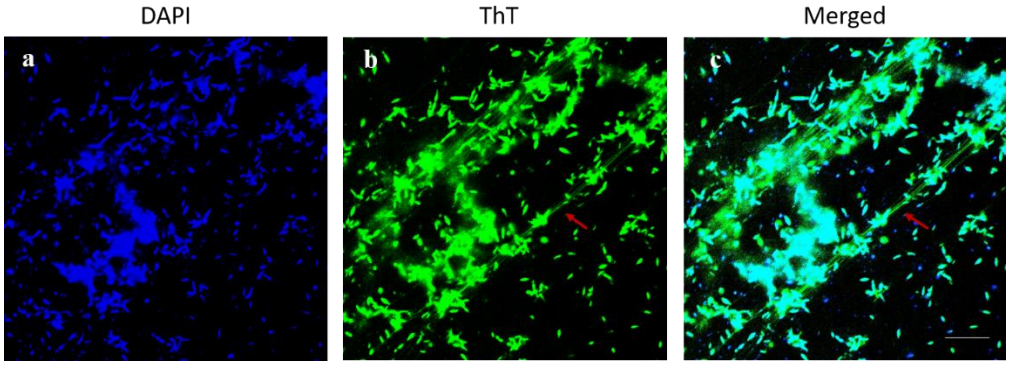


Fig 6. The mature biofilm of *C. jejuni* 11168-O and amyloid-like fibres. *C. jejuni* biofilm imaged using dual fluorescence labelling by CLSM. Red arrow indicates for amyloid-like *fibrils* (ThioT, green) and bacterial cells within the biofilm (DAPI, blue). (Scale bar= 10µm).

Expression level of *alr* and *ddlA* in the presence of LAs and DAs

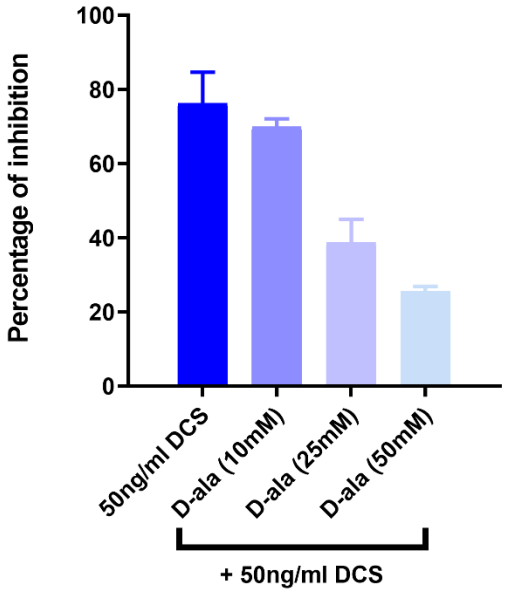
In order to interrogate the mechanism of inhibitory action of DAs and L-ala, the expression of *C. jejuni* PG biosynthesis enzymes alanine racemase (*alr*) and D-Ala-D-Ala ligase (*ddlA*) in the presence and absence of DAs and LAs were examined. The relative expression of *ddl* and *alr* was downregulated by 1.25 to 4-fold below the cut-off level, respectively, following treatment of cells with 25 mM of L-ala (Table 1). In contrast, 25 mM of D-ala upregulated the expression of *ddl* by 10-fold and *alr* by 38-fold. Treatment of cells with 25 mM D-trp downregulated the expression level of *ddl* by 1.65-fold and *alr* by 3-fold whereas D-ser (25 mM) downregulated the expression of *alr* by 2.92-fold and upregulated *ddl* by 2.58-fold. No significant effect on the expression of *alr* and *ddl* was observed following treatment with D-met (Table 1). Interestingly, treatment of cells with D-Cycloserine (DCS) (10ng/ml), as a positive control, had a greater effect, downregulating the expression of *ddlA* with a 7-fold change as compared to 2.85-fold change for *alr*. No loss of cell viability could be detected after 2-h exposure to DAs or DCS.

Table 1. Analysis of the relative expression of *alr* and *ddlA* genes in the present of LAs and DAs by real-time PCR (qRT-PCR). The relative expression of *alr* and *ddl* genes after incubation of *C. jejuni* 11168-O cells with 25 mM of LAs and DAs for 2 hrs.

	Fold change						
	upregulated			**downregulated**			
Gene name	D-ala	D-ser	D-met	L-ala	D-ser	D-trp	DCS
alr	38±7	-	-	4.18±0.3	2.92±0.2	1.64±0.3	2.85±0.2
ddlA	10±2	2.58±0.6	-	1.25±0.1	-	3.42±0.4	7.15±0.2

157 **D-Ala can reverse the inhibitory effect of DAs and DCS**

158 D-ala has been reported to reverse the antimicrobial efficacy of DCS in *Mycobacterium* spp
159 (38, 39). Considering that the MIC range of DCS for *Campylobacter* spp reported to be
160 between 0.25 μg/ml-4 μg/ml (40), we tested the effect of sub-inhibitory concentration of
161 50ng/ml DCS on *C. jejuni* cells and determined that DCS can reduce *C. jejuni* growth and
162 biofilm formation by up to 76% (Fig 7). Furthermore, this effect can be reversed by
163 increasing the concentration of D-ala from 10 mM to 50mM (Fig 7). Combining D-ala with
164 other DAs also decreased the inhibition of biofilm formation. In contrast, a combination of
165 DAs with DCS increased the efficacy of DCS at 10 ng/ml by 32% as compared with DCS
166 treatment alone (Fig 8).



167
168 **Fig 7.** Reversal of DCS growth inhibition by D-alanine at different concentrations in *C.*
169 *jejuni* 11168-O.

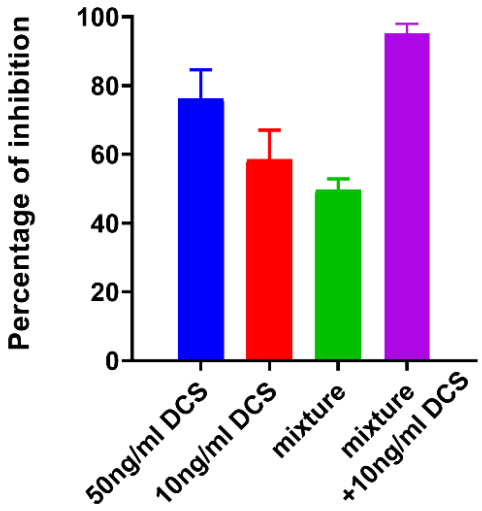


Fig 8. Effect of DCS on *C. jejuni* 11168-O biofilm when combined with L-ala, D-ser, D-met, D-trp (5:5:2:5 mM).

Discussion

This study describes the identification of specific small, naturally occurring molecules, DAs, which are highly effective in preventing and disrupting *C. jejuni* biofilms, in concert with that previously shown for *B. subtilis, S. aureus* and *P. aeruginosa* (24, 36, 41). While D-met and D-trp are able to inhibit the biofilm formation of *C. jejuni*, L-form of those amino acids significantly increased biofilm formation. It is possible that *C. jejuni* is able to catabolize L-form of those amino acids (42), which promotes bacterial growth, and consequently formation of the biofilm. This is consistent with the previous report of *B. subtilis* growth inhibition by D-form of Tyr, Leu, and Trp, and the L-form of those amino acids counteracting this effect (23). The effect of DAs on inhibition and dispersal of *C. jejuni* biofilms showed a concentration-dependent response, with D-ser, D-met and D-trp being most effective in inhibition and dispersion of the biofilm. We observed that D-met, and D-trp, have a significant dispersive effect on biofilms at concentrations of ≥10 mM, similar to that observed

for *S. aureus* and *P. aeruginosa* (43). It's important to note that, the inhibitory effect on the growth of *C. jejuni* by DAs, except D-met, could be reversed by D-ala, similar to that observed for *B. subtilis* , *M. tuberculosis* and *Escherichia coli* (38, 39, 44, 45).

Microscopic analysis confirmed the effect of DAs on biofilm formation of *C. jejuni,* and particularly, the formation of amyloid-like fibrils within the biofilm matrix. Matrix-associated amyloid fibrils had been previously reported to form a part of C. jejuni biofilm (46), and similar DA-induced disassembly of matrix-associated amyloid fibers of *B. subtilis* biofilm, had been proposed as a biofilm-dispersal mechanism (34, 41). Together, these data allow us to speculate that the ability of DAs to promote the dispersal of formed C. *jejuni* biofilms, could involve the triggering the disassembly of matrix-associated amyloid fibrils.

While the mechanisms of antimicrobial and antibiofilm action of DAs, particularly, D-ser, D-met, and D-trp, are not fully understood, DAs effect on *C. jejuni* growth and biofilm formation may be similar to that for *Alcaligenes faecalis*, where D-met incorporates into PG, causing morphological and structural damage to the cell wall (30, 37, 47), and consequently suppresses bacterial growth**.** To explore that possibility, we interrogated the effect of DAs and LAs on the expression level of two genes in *C. jejuni*; alanine racemase (*alr*) (*Cj0905c*), and D-Ala-D-Ala ligase (*ddlA*) (*Cj0798c*) (48, 49). Both genes are encoding enzymes involved in an important step in D-Ala metabolism (44, 50), which is essential for the synthesis of PG of the bacterial cell wall (45, 51, 52). Two main reactions are involved in this process, first the conversion of L-Ala to D-Ala by alanine racemase (alr), and the formation of D-alanyl–D-alanine dipeptide (D-Ala-D-Ala) from D-Ala by D-alanine–D-

alanine ligase (ddl) (53). RT-PCR data shows that DCS able to reduce both *C. jejuni alr* and *ddlA* expression levels, similarly to L-ala, and D-trp. Interestingly, D-ser reduced *alr* expression levels, but not that of *ddlA*, suggesting that *ddlA* may not be the primary target for D-ser or DCS in *C. jejuni*. Furthermore, the ability of D-ala to reverse the inhibitory effect of DCS and D-ser suggests that the inhibitory effect of DCS and D-ser on *C. jejuni* can be mediated through inhibition of *alr* alone. In contrast, in *M. tuberculosis,* both *alr* and *ddl* were reported to be the primary targets of DCS (39), and *S. Halouska, et al. (54)* suggested that *ddl* ay be a primary target of DCS, rather than *alr*.

It is interesting to note that bacterial PG dipeptide D-Ala-D-Ala, which is generated by D-Ala-D-Ala ligase (*ddlA*), is the usual target for vancomycin, but in *C. jejuni*, PG contains D-Alanyl-D-Lactate (D-Ala-D-Lac) termini resulting in reduced efficacy of vancomycin by up to 1,000-fold. Substitution by D-alanyl-D-serine (D-Ala-D-ser) termini reduces the efficacy of this antibiotic by up to 7-fold (4, 55-58). This further suggests that *alr* and not *ddlA*, is likely to be the primary target for D-ser and DCS in *C. jejuni*,.

Our results suggest that DAs might have a promising application in enhancing the activity antibiotics where the combination of DAs with DCS, synergistically increased the ability of DCS to inhibit *C. jejuni* biofilm formation and growth. The enhancement of DCS efficacy with DAs is likely to lower minimal dose requirement, which would consequently reduce the drug toxicity. DAs had also been reported to enhance the effectiveness for colistin and ciprofloxacin, when used against biofilms of *P. aeruginosa*, and rifampin used against biofilms of clinical isolates of *S. aureus* (43).

To summarize, this study suggests that (i) DAs show the inhibitory effect at millimolar concentrations on biofilm formation by C. *jejuni*; (ii) DAs can trigger C. *jejuni* biofilm-disassembly; (iii) a combination of DAs can enhance the efficacy of DSC, (iv) DAs inhibit growth and biofilm formation of *C. jejuni* by repressing the expression of *alr*. The data described here contribute to the understanding of the mechanisms involved in biofilm dispersion and inform on identification of potential antimicrobial drug targets.

Materials and Methods

***C. jejuni* strains and growth conditions.** Bacterial strains used in this study were *C. jejuni* 11168-O (courtesy of Prof. D. G. Newell, United Kingdom), *C. jejuni* 81-176 (courtesy of Prof. Christine Szymanski, University of Alberta, Alberta), and *C. coli* NCTC 11366 (Griffith University culture collection, Australia). Cells were grown at 42ºC microaerobically (85% N_2, 10% CO_2 and 5% O_2) on Mueller-Hinton agar (MHA) and in Mueller-Hinton broth (MHB), supplemented with Trimethoprim (5 μg ml^{-1}) and Vancomycin (10 μg ml^{-1}) (TV) (Sigma).

Chemical and reagents used in this study. L-alanine (L-ala), D-alanine (D-ala), L-serine (L-ser), D-serine (D-ser), L-methionine (L-met), D-methionine (D-met), L-tryptophan (L-trp), D-tryptophan (D-trp) D- cycloserine were from Sigma-Aldrich. Individual stock solutions of 100 mM of DAs in Phosphate-buffered saline (PBS) (PH 7.2).

Biofilm formation and dispersion assays. Overnight cultures of *C. jejuni* strains were diluted to an OD_{600} of 0.05, and 2 mL of cell suspension was placed into 24-wells flat-bottom polystyrene tissue culture plates (Geiner Bio-One). Different concentrations of DAs (1-100

mM) were added directly to the culture in the wells and incubated at 42°C under microaeroaerobic conditions for 48 hours. For dispersion assay, *C. jejuni* cells were grown as described above, except no DAs were added. Then PBS containing the appropriate concentration of DAs (0.1-100 mM) was added to the wells and plates incubated for further 24 hrs. For crystal violet staining, plates were rinsed with water once (gently) and dried at 55°C for 30 minutes and stained using modified crystal violet staining method as described previously (59). Data are representative of three independent experiments, and values are expressed in presented as Mean± S.D.

RNA extraction, cDNA synthesis and RT-qPCR of Alanine racemase (*alr*), D-alanine-D-alanine ligase (*ddlA*). *C. jejuni* 11168-O cells were grown overnight microaerobically in MHB at 42ºC. Cells were collected by centrifuging at 4000 rpm for 15 minutes. The pellets were suspended in MHB and OD_{600} adjusted to 1 (~3×10^9 cells/ml) and subsequently challenged with (1) 25 mM of L-ala, (2)25 mM of D-ala ,(3) 25 mM of D-ser, (4) 25 mM of D-met or ,(5) 25 mM of D-trp for 2-h; (5) 10ng/ml of DCS (below MIC which 250 ng/ml) was used as control. The bacterial survival was confirmed by viable cells counts after 2-h. Then, cells were collected by centrifugation at 4000 rpm for 15 minutes and pellets used for RNA extraction by RNeasy kit according to the manufacturer's protocol (Bioline). cDNA synthesis and RT-qPCR was performed as previously described (60). The following primers sets were used: *alr* (Cj0905c) forward 3-AGCCAAAAATTTAGGAGTTT-5 and *alr* reverse 5-GAGGACGATGTGATAGTATT-3, *ddl* (Cj0798c) forward 3-TTATTTTTTGTGATGAAGAAAGAA-5 and *sdl* reverse 5-GAGTTCTTTTTCTTTTTTATAAGC-3. A *gryA* gene was used as a housekeeping control gene, using the primers, *gryA* forward 3-CCACTGGTGGTGAAGAAAATTTA-5 and *gryA* reverse 5-AGCATTTTACCTTGTGTGCTTAC-3. Relative *n*-fold changes in the transcription of the examined genes between the treated and non-treated samples were

274 calculated using the relative quantification (RQ), also known as $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T$
275 = ΔC_T(treated sample) − ΔC_T(untreated sample), ΔC_T = C_T(target gene) − C_T(*gyrA*), and C_T
276 is the threshold cycle value for the amplified gene. The fold change due to treatment was
277 calculated as $-1/2^{-\Delta\Delta CT}$ (61, 62). The data are presented as Mean± S.D and were calculated
278 from triplicate cultures and are representative of three independent experiments.

279 **Confocal laser scanning microscopy.** Overnight cultures of *C. jejuni* cells were diluted to
280 an OD_{600} of 0.05, and 3 mL of each sample was placed into duplicate wells of a 6-well flat-
281 bottom polystyrene tissue culture plate containing a glass coverslip to enable the formation
282 of biofilm (Geiner Bio-One). 25 mM of LAs and DAs were added directly to the wells, and
283 then the plates were incubated at 42°C microaerobically for 48 hours. After the incubation,
284 MH broth was removed, and the wells were gently washed with PBS solution twice to remove
285 planktonic cells. The coverslips were carefully removed by using sterile needle and forceps
286 to new 6-well plates and fixed using 5% formaldehyde solution for 1 h at room temperature.
287 Then, the coverslips were gently washed with 2 mL of PBS and prepared for staining with
288 fluorescent dyes.

289 **Staining of *C. jejuni* cells.** The fluorescent DNA-binding stain DAPI (Sigma Aldrich) was
290 used to visualise cell distribution as described previously (63). Thioflavin T (ThT) at 20 µM
291 was then used to treat the coverslips for 30 minutes. ThT emits green fluorescence upon
292 binding to cellulose or amyloids (64, 65). The coverslips then were mounted on glass slides
293 using the mounting medium (Ibidi GmbH, Martinsried, Germany) and sealed with
294 transparent nail varnish. Microscopy (Nikon A1R+) (Griffith University) was performed with
295 two coverslips per sample from at least two separate experiments. All images were processed

using ImageJ analysis software version 1.5i (National Institutes of Health, Besthda, Maryland).

Statistical analysis. The statistical analyses performed using GraphPad Prism, version 6.00 (for Windows; GraphPad Software) to calculate statistically significant differences when *P*-value by applied Student's *t*-test.

Author Contributions:

VK and BE conceived and designed the study; BE and Taha performed the experiments; VK, and BE, analyzed the data and prepared the manuscript. All authors reviewed the manuscript.

Acknowledgements:

The authors thank Neeraj Bhatnagar, Michael Hadjistyllis for technical assistance.

Funding:

This project was supported by Griffith University, Institute for Glycomics.

Conflicts of Interest:

Authors declare that there is no conflict of interest regarding the publication of this article.

316 **References**

317 1. **Grantham-McGregor S, Cheung YB, Cueto S, Glewwe P, Richter L, Strupp B,**
318 **Group ICDS.** 2007. Developmental potential in the first 5 years for children in
319 developing countries. The lancet **369:**60-70.
320 2. **WHO WHO.** 2012. The global view of campylobacteriosis: report of an expert
321 consultation. World Health Organization, Utrecht, The Netherlands.
322 3. **Luangtongkum T, Jeon B, Han J, Plummer P, Logue CM, Zhang Q.** 2009.
323 Antibiotic resistance in Campylobacter: emergence, transmission and persistence.
324 4. **Iovine NM.** 2013. Resistance mechanisms in *Campylobacter jejuni*. Virulence
325 **4:**230-240.
326 5. **Engberg J, Aarestrup FM, Taylor DE, Gerner-Smidt P, Nachamkin I.** 2001.
327 Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance
328 mechanisms and trends in human isolates. Emerg Infect Dis **7:**24.
329 6. **Ica T, Caner V, Istanbullu O, Nguyen HD, Ahmed B, Call DR, Beyenal H.** 2012.
330 Characterization of mono- and mixed-culture *Campylobacter jejuni* biofilms. Appl
331 Environ Microbiol **78:**1033-1038.
332 7. **Zimmer M, Barnhart H, Idris U, Lee MD.** 2003. Detection of *Campylobacter*
333 *jejuni* strains in the water lines of a commercial broiler house and their relationship to the
334 strains that colonized the chickens. Avian Dis **47:**101-107.
335 8. **Joshua GP, Guthrie-Irons C, Karlyshev A, Wren B.** 2006. Biofilm formation in
336 *Campylobacter jejuni*. Microbiology **152:**387-396.
337 9. **Bronowski C, James CE, Winstanley C.** 2014. Role of environmental survival in
338 transmission of *Campylobacter jejuni*. FEMS Microbiol Lett **356:**8-19.

10. **Moore JE, Barton MD, Blair IS, Corcoran D, Dooley JS, Fanning S, Kempf I, Lastovica AJ, Lowery CJ, Matsuda M.** 2006. The epidemiology of antibiotic resistance in Campylobacter. Microbes and Infection **8:**1955-1966.

11. **Smith JL, Fratamico PM.** 2010. Fluoroquinolone resistance in Campylobacter. Journal of Food Protection® **73:**1141-1152.

12. **Organization WH.** 2017. WHO publishes list of bacteria for which new antibiotics are urgently needed.

13. **Alfredson DA, Korolik V.** 2007. Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. FEMS Microbiol Lett **277:**123-132.

14. **Miflin JK, Templeton JM, Blackall P.** 2007. Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East Queensland region. J Antimicrob Chemother **59:**775-778.

15. **Tambur Z, Miljković-Selimović B, Bokonjić D.** 2009. Determination of sensitivity to antibiotics of *Campilobacter* jejuni and Campilobacter coli isolated from human feces. Vojnosanit Pregl **66:**49-52.

16. **Sharma D, Misba L, Khan AU.** 2019. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrobial Resistance & Infection Control **8:**76.

17. **Kaplan JB.** 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J Dent Res **89:**205-218.

18. **Sauer K, Cullen MC, Rickard AH, Zeef LAH, Davies DG, Gilbert P.** 2004. Characterization of Nutrient-Induced Dispersion in Pseudomonas aeruginosa PAO1 Biofilm. J Bacteriol **186:**7312-7326.

19. **Rumbaugh KP.** 2014. Antibiofilm Agents: From Diagnosis to Treatment and Prevention, vol 8. Springer Science & Business Media.

20. **Nahar S, Mizan MFR, Ha AJw, Ha SD.** 2018. Advances and future prospects of enzyme-based biofilm prevention approaches in the food industry. Comprehensive Reviews in Food Science and Food Safety **17:**1484-1502.

21. **Brandenburg KS, Rodriguez KJ, McAnulty JF, Murphy CJ, Abbott NL, Schurr MJ, Czuprynski CJ.** 2013. Tryptophan inhibits biofilm formation by *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **57:**1921-1925.

22. **Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R.** 2013. Sticking together: building a biofilm the *Bacillus subtilis* way. Nat Rev Microbiol **11:**157-168.

23. **Leiman SA, May JM, Lebar MD, Kahne D, Kolter R, Losick R.** 2013. d-Amino acids indirectly inhibit biofilm formation in *Bacillus subtilis* by interfering with protein synthesis. J Bacteriol **195:**5391-5395.

24. **Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, Losick R.** 2012. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell **149:**684-692.

25. **Xu H, Liu Y.** 2011. Reduced microbial attachment by d-amino acid-inhibited AI-2 and EPS production. Water research **45:**5796-5804.

26. **Zilm PS, Butnejski V, Rossi-Fedele G, Kidd SP, Edwards S, Vasilev K.** 2017. D-amino acids reduce *Enterococcus faecalis* biofilms in vitro and in the presence of antimicrobials used for root canal treatment. PloS one **12**.

27. **Aliashkevich A, Alvarez L, Cava F.** 2018. New insights into the mechanisms and biological roles of d-amino acids in complex eco-systems. Frontiers in microbiology **9:**683.

28. **Azúa I, Goiriena I, Baña Z, Iriberri J, Unanue M.** 2014. Release and consumption of D-amino acids during growth of marine prokaryotes. Microb Ecol **67:**1-12.

29. **Rendueles O, Ghigo J-M.** 2012. Multi-species biofilms: how to avoid unfriendly neighbors. FEMS Microbiol Rev **36:**972-989.

30. **Lam H, Oh DC, Cava F, Takacs CN, Clardy J, de Pedro MA, Waldor MK.** 2009. D-amino acids govern stationary phase cell wall remodeling in bacteria. Science **325:**1552-1555.

31. **Flemming H-C, Wingender J.** 2010. The biofilm matrix. Nat Rev Micro **8:**623-633.

32. **Kostakioti M, Hadjifrangiskou M, Hultgren SJ.** 2013. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic Era. Cold Spring Harbor Perspectives in Medicine **3**.

33. **Zhang Z, Li Z, Jiao N.** 2014. Effects of D-amino acids on the EPS production and cell aggregation of *Alteromonas macleodii* strain JL2069. Curr Microbiol **68:**751-755.

34. **Cava F, Lam H, de Pedro MA, Waldor MK.** 2011. Emerging knowledge of regulatory roles of D-amino acids in bacteria. Cell Mol Life Sci **68:**817-831.

35. **Ramon-Perez ML, Diaz-Cedillo F, Ibarra JA, Torales-Cardena A, Rodriguez-Martinez S, Jan-Roblero J, Cancino-Diaz ME, Cancino-Diaz JC.** 2014. D-Amino acids inhibit biofilm formation in *Staphylococcus epidermidis* strains from ocular infections. J Med Microbiol **63:**1369-1376.

36. **Hochbaum AI, Kolodkin-Gal I, Foulston L, Kolter R, Aizenberg J, Losick R.** 2011. Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. J Bacteriol **193:**5616-5622.

37. **Lark C, Lark KG.** 1961. Studies on the mechanism by which D-amino acids block cell wall synthesis. Biochim Biophys Acta **49:**308-322.

412 38. **Moulder JW, Novosel DL, Officer JE.** 1963. Inhibition of the growth of agents of
413 the psittacosis group by D-cycloserine and its specific reversal by D-alanine. J Bacteriol
414 **85:**707-711.
415 39. **Awasthy D, Bharath S, Subbulakshmi V, Sharma U.** 2012. Alanine racemase
416 mutants of *Mycobacterium tuberculosis* require D-alanine for growth and are defective
417 for survival in macrophages and mice. Microbiology **158:**319-327.
418 40. **Samie A, Ramalivhana J, Igumbor E, Obi C.** 2007. Prevalence, haemolytic and
419 haemagglutination activities and antibiotic susceptibility profiles of *Campylobacter* spp.
420 isolated from human diarrhoeal stools in Vhembe District, South Africa. Journal of
421 health, population, and nutrition **25:**406.
422 41. **Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R.** 2010. D-amino
423 acids trigger biofilm disassembly. Science **328:**627-629.
424 42. **van der Hooft JJ, Alghefari W, Watson E, Everest P, Morton FR, Burgess KE,**
425 **Smith DG.** 2018. Unexpected differential metabolic responses of *Campylobacter jejuni*
426 to the abundant presence of glutamate and fucose. Metabolomics **14:**144.
427 43. **Sanchez CJ, Akers KS, Romano DR, Woodbury RL, Hardy SK, Murray CK,**
428 **Wenke JC.** 2014. d-Amino acids enhance the activity of antimicrobials against biofilms
429 of clinical wound isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.
430 Antimicrob Agents Chemother **58:**4353-4361.
431 44. **Wijsman H.** 1972. The characterization of an alanine racemase mutant of
432 *Escherichia coli*. Genetics Research **20:**269-277.
433 45. **Sidiq KR, Chow M, Zhao Z, Daniel R.** 2019. Alanine Metabolism in *Bacillus*
434 *subtilis*. bioRxiv**:**562850.

435 46. **Turonova H, Neu TR, Ulbrich P, Pazlarova J, Tresse O.** 2016. The biofilm
436 matrix of *Campylobacter jejuni* determined by fluorescence lectin-binding analysis.
437 Biofouling **32:**597-608.
438 47. **Cava F, De Pedro MA, Lam H, Davis BM, Waldor MK.** 2011. Distinct pathways
439 for modification of the bacterial cell wall by non-canonical D-amino acids. The EMBO
440 journal **30:**3442-3453.
441 48. **Mandal RK, Jiang T, Kwon YM.** 2017. Essential genome of *Campylobacter*
442 *jejuni*. BMC genomics **18:**616.
443 49. **Gao B, Lara-Tejero M, Lefebre M, Goodman AL, Galán JE.** 2014. Novel
444 components of the flagellar system in epsilonproteobacteria. MBio **5:**e01349-01314.
445 50. **Chacon O, Bermudez LE, Zinniel DK, Chahal HK, Fenton RJ, Feng Z,**
446 **Hanford K, Adams LG, Barletta RG.** 2009. Impairment of D-alanine biosynthesis in
447 *Mycobacterium smegmatis* determines decreased intracellular survival in human
448 macrophages. Microbiology **155:**1440-1450.
449 51. **Qiu W, Zheng X, Wei Y, Zhou X, Zhang K, Wang S, Cheng L, Li Y, Ren B, Xu**
450 **X.** 2016. d-Alanine metabolism is essential for growth and biofilm formation of
451 *Streptococcus mutans*. Molecular oral microbiology **31:**435-444.
452 52. **Wei Y, Qiu W, Zhou X-D, Zheng X, Zhang K-K, Wang S-D, Li Y-Q, Cheng L,**
453 **Li J-Y, Xu X.** 2016. Alanine racemase is essential for the growth and interspecies
454 competitiveness of *Streptococcus mutans*. International journal of oral science **8:**231.
455 53. **Batson S, Rea D, Fülöp V, Roper DI.** 2010. Crystallization and preliminary X-ray
456 analysis of a D-alanyl-D-alanine ligase (EcDdlB) from *Escherichia coli*. Acta
457 Crystallographica Section F: Structural Biology and Crystallization Communications
458 **66:**405-408.

459 54. **Halouska S, Fenton RJ, Zinniel DK, Marshall DD, Barletta RlG, Powers R.**
460 2014. Metabolomics analysis identifies d-alanine-d-alanine ligase as the primary lethal
461 target of d-cycloserine in mycobacteria. Journal of proteome research **13:**1065-1076.
462 55. **Van Der Aart LT, Lemmens N, van Wamel WJ, van Wezel GP.** 2016. Substrate
463 inhibition of VanA by D-alanine reduces vancomycin resistance in a VanX-dependent
464 manner. Antimicrob Agents Chemother **60:**4930-4939.
465 56. **Healy VL, Lessard IA, Roper DI, Knox JR, Walsh CT.** 2000. Vancomycin
466 resistance in enterococci: reprogramming of the d-Ala–d-Ala ligases in bacterial
467 peptidoglycan biosynthesis. Chem Biol **7:**R109-R119.
468 57. **Lessard IA, Healy VL, Park I-S, Walsh CT.** 1999. Determinants for differential
469 effects on d-Ala-d-lactate vs d-Ala-d-Ala formation by the VanA ligase from
470 vancomycin-resistant enterococci. Biochemistry (Mosc) **38:**14006-14022.
471 58. **Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S,**
472 **Leclercq R, Courvalin P, Cattoir V.** 2011. D-Ala-D-Ser VanN-type transferable
473 vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother
474 **55:**4606-4612.
475 59. **Tram G, Korolik V, Day CJ.** 2013. MBDS solvent: an improved method for
476 assessment of biofilms. Advances in Microbiology **3:**5.
477 60. **Day, Hartley-Tassell LE, Shewell LK, King RM, Tram G, Day SK, Semchenko**
478 **EA, Korolik V.** 2012. Variation of chemosensory receptor content of *Campylobacter*
479 *jejuni* strains and modulation of receptor gene expression under different in vivo and in
480 vitro growth conditions. BMC Microbiol **12:**128.
481 61. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative
482 CT method. Nature protocols **3:**1101-1108.

62. **Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H.** 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C T difference" formula. J Mol Med **84:**901-910.

63. **Peeters E, Nelis HJ, Coenye T.** 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods **72:**157-165.

64. **Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK, Roy R, Singh S.** 2005. Mechanism of thioflavin T binding to amyloid fibrils. J Struct Biol **151:**229-238.

65. **Biancalana M, Koide S.** 2010. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics **1804:**1405-1412.