

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

25 **Introduction**

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

44 (27, 28), yet naturally occurring DAs have been found in different environments, such as soil,
45 as well as in human and animals tissues (27). In addition, many bacterial species secrete DAs
46 in the stationary growth phase and when encased in biofilms. For example, *Vibrio cholerae*
47 can produce D-methionine (D-met) and D-leucine (D-leu), while *B. subtilis* generates D-
48 tyrosine (D-tyr) and D-phenylalanine (D-phe) which can accumulate at millimolar
49 concentrations (29, 30). The ability of bacteria to produce DAs is proposed to be a mechanism
50 for self-dispersal of aging biofilms, and DA production may also inhibit the growth of other
51 bacteria during maturation of mixed biofilms. In a naturally occurring biofilms, DAs are
52 found to be involved in the regulation of extracellular polymeric saccharide (EPS)
53 production, for instance, D-tyr reduces the attachment of *B. subtilis*, *S. aureus* and *P.*
54 *aeruginosa* to surfaces (22, 24, 31-33). Also, DAs can induce disassembly of matrix-
55 associated amyloid fibrils that link the cells within the biofilm and contribute to the biofilm
56 strength (34). Effective concentration of DAs required to inhibit the biofilm formation varies
57 depending on bacterial strain and DAs concentration ranging between 3 μ M and 10 mM (23,
58 33, 35, 36). It's important to note that some DAs exhibit inhibitory or toxic effects on a
59 number of bacterial species and can interfere with the activities of peptidases and proteases
60 involved in cell wall synthesis, for example, D-met can be incorporated into the
61 peptidoglycan (PG) of bacterial cell walls, causing morphological and structural damage
62 (37).

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

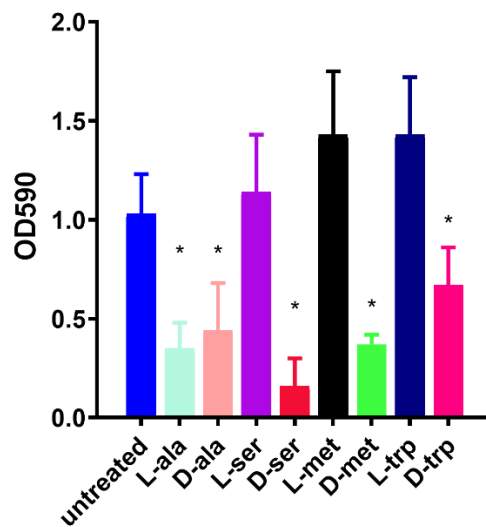
66 Herein, we demonstrate that D-alanine (D-ala), L-alanine (L-ala), D-serine (D-ser), D-
67 methionine (D-met), and D-tryptophan (D-trp) can inhibit and disperse biofilms formed by
68 *C. jejuni* and *C. coli* and that it may be possible to use these DAs to enhance the efficacy of
69 antibiotics such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

73 **Results**

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

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

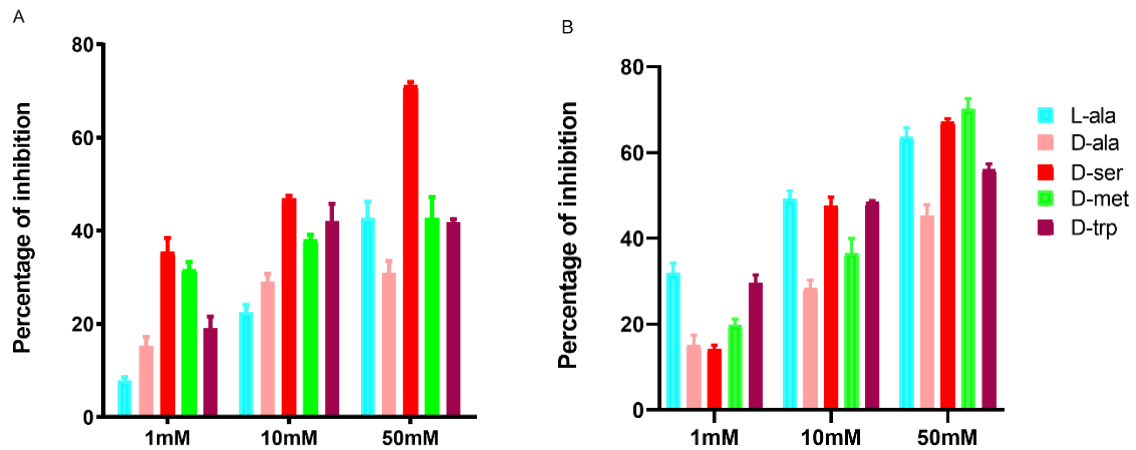
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87 **Fig 1.** Effect of 100 mM DAs and LAs on *C. jejuni* 11168-O biofilm. Inhibition of biofilm
88 formation in the presence of 100 mM of; L-alanine (L-ala), D- alanine (D-ala), L-serine (L-
89 ser), D-serine (D-ser), L-methionine (L-met), D-methionine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

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

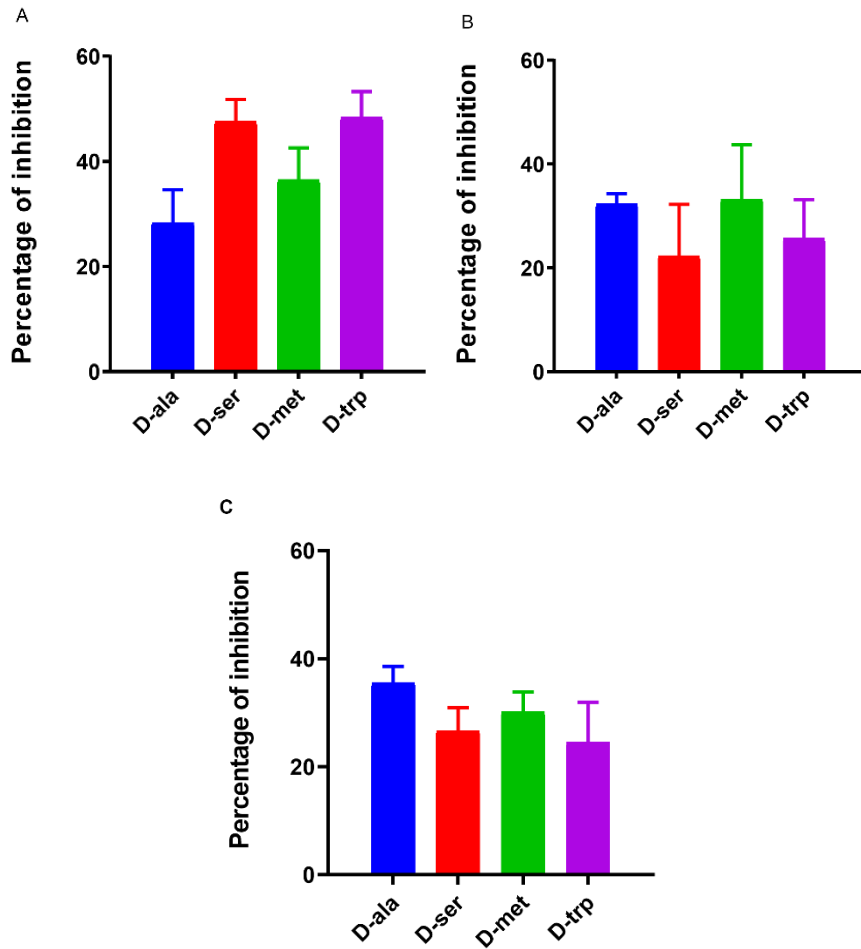


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101 **Fig 2.** Inhibition and dispersion response of *C. jejuni* 11168-O biofilms in the presence of
102 LAs and DAs at different concentrations. A) Dispersion of the existing biofilm induced by
103 different concentrations of LAs and DAs. B) Inhibition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

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

110 The equimolar mixture of DAs and LAs (1:1) showed $\geq 40\%$ inhibition of *C. jejuni* 11168-
111 O biofilm formation (Fig 4). The mixture of the four amino acids, L-ala, D-met, D-ser, D-trp
112 (5:5:2:5 mM), was more potent, with up to 49% inhibition of biofilm formation; however,
113 the addition of D-ala to D-ser decreased the inhibitory effect (Fig 4).



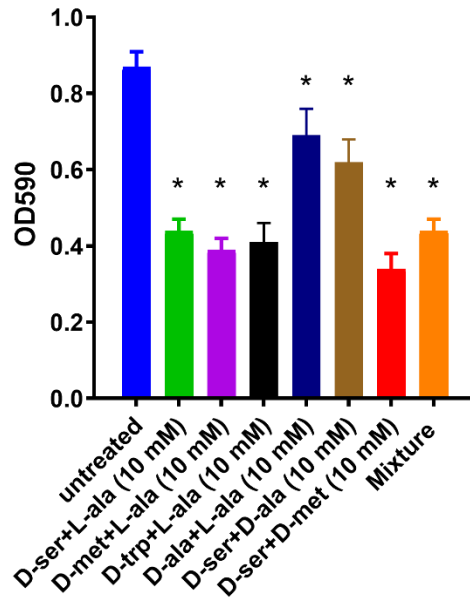
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116 **Fig 3.** Quantitative analysis of biofilm inhibition of A) *C. jejuni* 11168-O, B) *C. jejuni* 81-

117 176, and C) *C. coli* NCTC 11366 in the presence of 10 mM of DAs.

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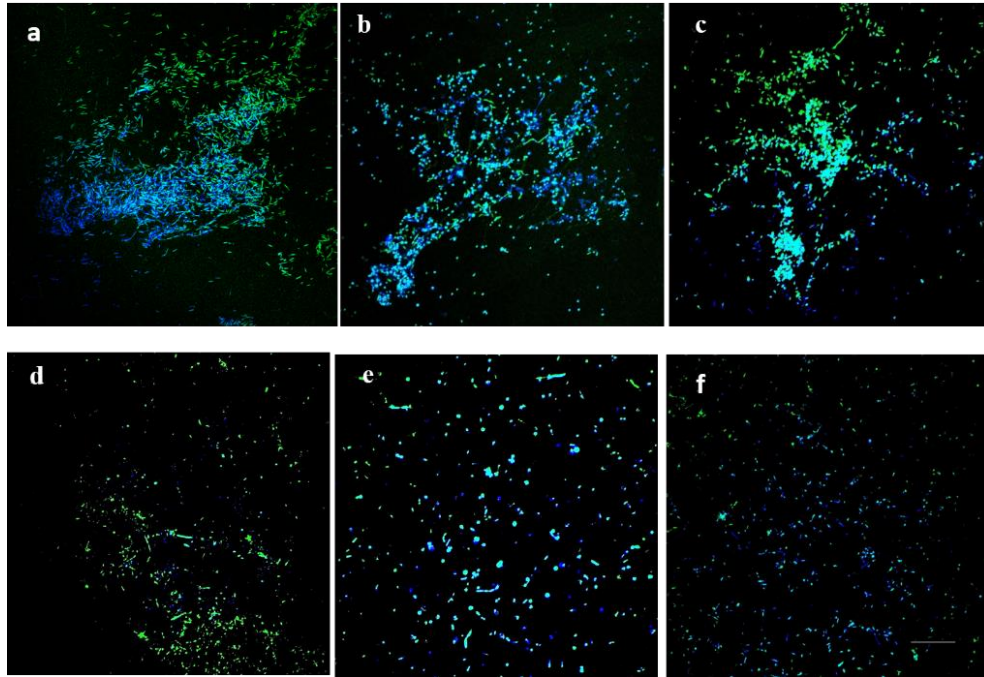
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120 **Fig 4.** Effect of the equimolar mixture of DAs and LAs on *C. jejuni* 11168-O biofilm. The
121 asterisk (*) indicates a statistically significant difference using the unpaired Student's t-test,
122 $p < 0.05$.

123

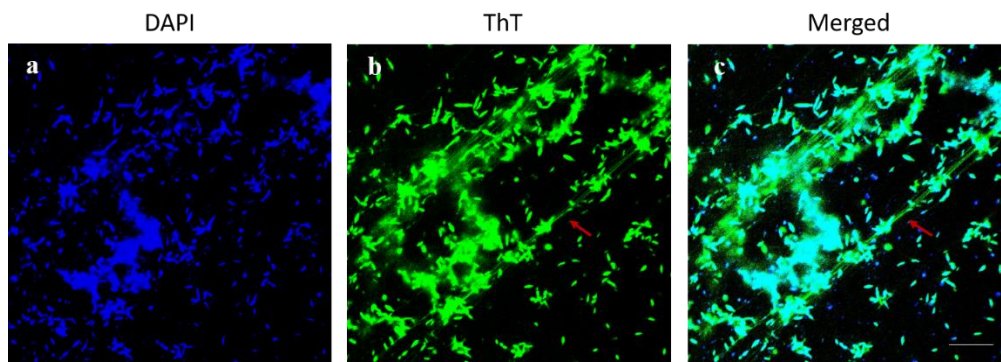
124 **Microscopic characterization of the dispersion effect of DAs on biofilm formation**

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



129

130 **Fig 5.** Confocal scanning laser microscopy images of *C. jejuni* 11168-O biofilm in presence
131 of 25 mM of DAs. *C. jejuni* biofilm at 48h, imaged using dual fluorescence labelling by
132 CLSM. a) Untreated, b) D-ala, c) L-ala, d) D-ser, e) D-met, f) D-trp. Cells were stained with
133 4',6-diamidino-2-phenylindole (DAPI, blue) and amyloid fibrils by Thioflavin T (ThioT,
134 green) (Scale bar= 20 μ m).



135

136 **Fig 6.** The mature biofilm of *C. jejuni* 11168-O and amyloid-like fibres. *C. jejuni* biofilm
137 imaged using dual fluorescence labelling by CLSM. Red arrow indicates for amyloid-like
138 *fibrils* (ThioT, green) and bacterial cells within the biofilm (DAPI, blue). (Scale bar= 10 μ m).
139

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

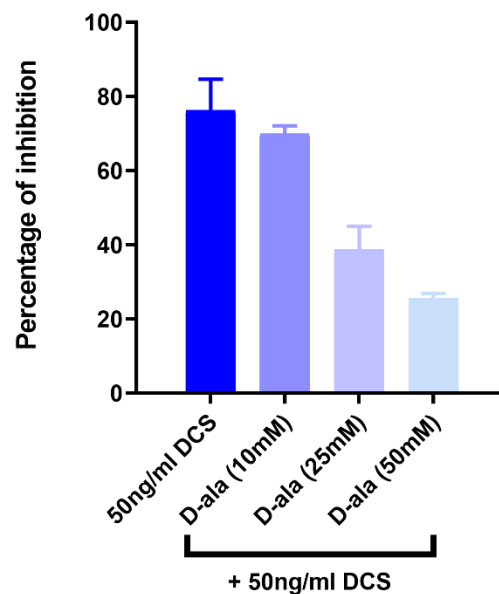
141 In order to interrogate the mechanism of inhibitory action of DAs and L-ala, the expression
 142 of *C. jejuni* PG biosynthesis enzymes alanine racemase (*alr*) and D-Ala-D-Ala ligase (*ddlA*)
 143 in the presence and absence of DAs and LAs were examined. The relative expression of *ddl*
 144 and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

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

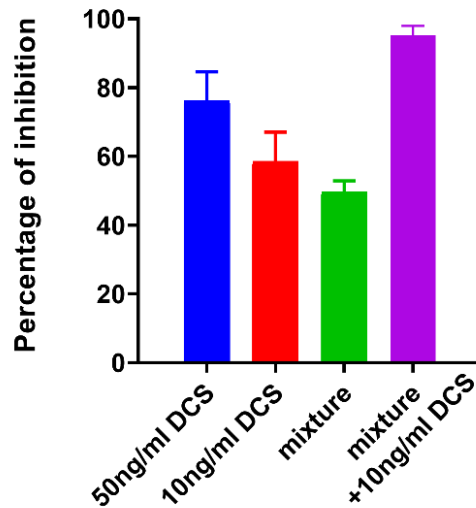
Gene name	Fold change						
	upregulated			downregulated			
	D-ala	D-ser	D-met	L-ala	D-ser	D-trp	DCS
<i>alr</i>	38±7	-	-	4.18±0.3	2.92±0.2	1.64±0.3	2.85±0.2
<i>ddlA</i>	10±2	2.58±0.6	-	1.25±0.1	-	3.42±0.4	7.15±0.2

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



170
171 **Fig 8.** Effect of DCS on *C. jejuni* 11168-O biofilm when combined with L-ala, D-ser, D-met,
172 D-trp (5:5:2:5 mM).

173 Discussion

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

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

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

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

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

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

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

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

234 **Materials and Methods**

235 ***C. jejuni* strains and growth conditions.** Bacterial strains used in this study were *C. jejuni*
236 11168-O (courtesy of Prof. D. G. Newell, United Kingdom), *C. jejuni* 81-176 (courtesy of
237 Prof. Christine Szymanski, University of Alberta, Alberta), and *C. coli* NCTC 11366
238 (Griffith University culture collection, Australia). Cells were grown at 42°C microaerobically
239 (85% N₂, 10% CO₂ and 5% O₂) on Mueller-Hinton agar (MHA) and in Mueller-Hinton broth
240 (MHB), supplemented with Trimethoprim (5 µg ml⁻¹) and Vancomycin (10 µg ml⁻¹) (TV)
241 (Sigma).

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

246 **Biofilm formation and dispersion assays.** Overnight cultures of *C. jejuni* strains were
247 diluted to an OD₆₀₀ of 0.05, and 2 mL of cell suspension was placed into 24-wells flat-bottom
248 polystyrene tissue culture plates (Geiner Bio-One). Different concentrations of DAs (1-100

249 mM) were added directly to the culture in the wells and incubated at 42°C under
250 microaerobic conditions for 48 hours. For dispersion assay, *C. jejuni* cells were grown
251 as described above, except no DAs were added. Then PBS containing the appropriate
252 concentration of DAs (0.1-100 mM) was added to the wells and plates incubated for further
253 24 hrs. For crystal violet staining, plates were rinsed with water once (gently) and dried at
254 55°C for 30 minutes and stained using modified crystal violet staining method as described
255 previously (59). Data are representative of three independent experiments, and values are
256 expressed in presented as Mean± S.D.

257 **RNA extraction, cDNA synthesis and RT-qPCR of Alanine racemase (*alr*), D-alanine-**
258 **D-alanine ligase (*ddlA*).** *C. jejuni* 11168-O cells were grown overnight microaerobically in
259 MHB at 42°C. Cells were collected by centrifuging at 4000 rpm for 15 minutes. The pellets
260 were suspended in MHB and OD₆₀₀ adjusted to 1 (~3×10⁹ cells/ml) and subsequently
261 challenged with (1) 25 mM of L-ala, (2)25 mM of D-ala ,(3) 25 mM of D-ser, (4) 25 mM of
262 D-met or ,(5) 25 mM of D-trp for 2-h; (5) 10ng/ml of DCS (below MIC which 250 ng/ml)
263 was used as control. The bacterial survival was confirmed by viable cells counts after 2-h.
264 Then, cells were collected by centrifugation at 4000 rpm for 15 minutes and pellets used for
265 RNA extraction by RNeasy kit according to the manufacturer's protocol (Qiagen). cDNA
266 synthesis and RT-qPCR was performed as previously described (60). The following primers
267 sets were used: *alr* (Cj0905c) forward 3-AGCCAAAAATTTAGGAGTTT-5 and *alr* reverse
268 5-GAGGACGATGTGATAGTATT-3, *ddl* (Cj0798c) forward 3-
269 TTATTTTTTGTGATGAAGAAAGAA-5 and *sdl* reverse 5-
270 GAGTTCTTTTTCTTTTTTATAAGC-3. A *gryA* gene was used as a housekeeping control
271 gene, using the primers, *gryA* forward 3-CCACTGGTGGTGAAGAAAATTTA-5 and *gryA*
272 reverse 5-AGCATTTTACCTTGTGTGCTTAC-3. Relative *n*-fold changes in the
273 transcription of the examined genes between the treated and non-treated samples were

274 calculated using the relative quantification (RQ), also known as $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T$
275 = $\Delta C_T(\text{treated sample}) - \Delta C_T(\text{untreated sample})$, $\Delta C_T = C_T(\text{target gene}) - C_T(\text{gyrA})$, and C_T
276 is the threshold cycle value for the amplified gene. The fold change due to treatment was
277 calculated as $-1/2^{-\Delta\Delta C_T}$ (61, 62). The data are presented as Mean \pm 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 OD₆₀₀ 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 μ M
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

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

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

301 **Author Contributions:**

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

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309 **Conflicts of Interest:**

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

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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, Istanbulu 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.

- 339 10. **Moore JE, Barton MD, Blair IS, Corcoran D, Dooley JS, Fanning S, Kempf I,**
340 **Lastovica AJ, Lowery CJ, Matsuda M.** 2006. The epidemiology of antibiotic resistance
341 in *Campylobacter*. *Microbes and Infection* **8**:1955-1966.
- 342 11. **Smith JL, Fratamico PM.** 2010. Fluoroquinolone resistance in *Campylobacter*.
343 *Journal of Food Protection*® **73**:1141-1152.
- 344 12. **Organization WH.** 2017. WHO publishes list of bacteria for which new antibiotics
345 are urgently needed.
- 346 13. **Alfredson DA, Korolik V.** 2007. Antibiotic resistance and resistance mechanisms
347 in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol Lett* **277**:123-132.
- 348 14. **Miflin JK, Templeton JM, Blackall P.** 2007. Antibiotic resistance in
349 *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East
350 Queensland region. *J Antimicrob Chemother* **59**:775-778.
- 351 15. **Tambur Z, Miljković-Selimović B, Bokonjić D.** 2009. Determination of
352 sensitivity to antibiotics of *Campylobacter jejuni* and *Campylobacter coli* isolated from
353 human feces. *Vojnosanit Pregl* **66**:49-52.
- 354 16. **Sharma D, Misba L, Khan AU.** 2019. Antibiotics versus biofilm: an emerging
355 battleground in microbial communities. *Antimicrobial Resistance & Infection Control*
356 **8**:76.
- 357 17. **Kaplan JB.** 2010. Biofilm dispersal: mechanisms, clinical implications, and
358 potential therapeutic uses. *J Dent Res* **89**:205-218.
- 359 18. **Sauer K, Cullen MC, Rickard AH, Zeef LAH, Davies DG, Gilbert P.** 2004.
360 Characterization of Nutrient-Induced Dispersion in *Pseudomonas aeruginosa* PAO1
361 Biofilm. *J Bacteriol* **186**:7312-7326.
- 362 19. **Rumbaugh KP.** 2014. Antibiofilm Agents: From Diagnosis to Treatment and
363 Prevention, vol 8. Springer Science & Business Media.

- 364 20. **Nahar S, Mizan MFR, Ha AJw, Ha SD.** 2018. Advances and future prospects of
365 enzyme-based biofilm prevention approaches in the food industry. *Comprehensive*
366 *Reviews in Food Science and Food Safety* **17**:1484-1502.
- 367 21. **Brandenburg KS, Rodriguez KJ, McAnulty JF, Murphy CJ, Abbott NL,**
368 **Schurr MJ, Czuprynski CJ.** 2013. Tryptophan inhibits biofilm formation by
369 *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **57**:1921-1925.
- 370 22. **Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R.** 2013. Sticking together:
371 building a biofilm the *Bacillus subtilis* way. *Nat Rev Microbiol* **11**:157-168.
- 372 23. **Leiman SA, May JM, Lebar MD, Kahne D, Kolter R, Losick R.** 2013. d-Amino
373 acids indirectly inhibit biofilm formation in *Bacillus subtilis* by interfering with protein
374 synthesis. *J Bacteriol* **195**:5391-5395.
- 375 24. **Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, Losick R.** 2012.
376 A self-produced trigger for biofilm disassembly that targets exopolysaccharide. *Cell*
377 **149**:684-692.
- 378 25. **Xu H, Liu Y.** 2011. Reduced microbial attachment by d-amino acid-inhibited AI-2
379 and EPS production. *Water research* **45**:5796-5804.
- 380 26. **Zilm PS, Butnejski V, Rossi-Fedele G, Kidd SP, Edwards S, Vasilev K.** 2017.
381 D-amino acids reduce *Enterococcus faecalis* biofilms in vitro and in the presence of
382 antimicrobials used for root canal treatment. *PloS one* **12**.
- 383 27. **Aliashkevich A, Alvarez L, Cava F.** 2018. New insights into the mechanisms and
384 biological roles of d-amino acids in complex eco-systems. *Frontiers in microbiology*
385 **9**:683.
- 386 28. **Azúa I, Goiriena I, Baña Z, Iriberry J, Unanue M.** 2014. Release and
387 consumption of D-amino acids during growth of marine prokaryotes. *Microb Ecol* **67**:1-
388 12.

- 389 29. **Rendueles O, Ghigo J-M.** 2012. Multi-species biofilms: how to avoid unfriendly
390 neighbors. *FEMS Microbiol Rev* **36**:972-989.
- 391 30. **Lam H, Oh DC, Cava F, Takacs CN, Clardy J, de Pedro MA, Waldor MK.**
392 2009. D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science*
393 **325**:1552-1555.
- 394 31. **Flemming H-C, Wingender J.** 2010. The biofilm matrix. *Nat Rev Micro* **8**:623-
395 633.
- 396 32. **Kostakioti M, Hadjifrangiskou M, Hultgren SJ.** 2013. Bacterial biofilms:
397 development, dispersal, and therapeutic strategies in the dawn of the postantibiotic Era.
398 *Cold Spring Harbor Perspectives in Medicine* **3**.
- 399 33. **Zhang Z, Li Z, Jiao N.** 2014. Effects of D-amino acids on the EPS production and
400 cell aggregation of *Alteromonas macleodii* strain JL2069. *Curr Microbiol* **68**:751-755.
- 401 34. **Cava F, Lam H, de Pedro MA, Waldor MK.** 2011. Emerging knowledge of
402 regulatory roles of D-amino acids in bacteria. *Cell Mol Life Sci* **68**:817-831.
- 403 35. **Ramon-Perez ML, Diaz-Cedillo F, Ibarra JA, Torales-Cardena A, Rodriguez-**
404 **Martinez S, Jan-Roblero J, Cancino-Diaz ME, Cancino-Diaz JC.** 2014. D-Amino
405 acids inhibit biofilm formation in *Staphylococcus epidermidis* strains from ocular
406 infections. *J Med Microbiol* **63**:1369-1376.
- 407 36. **Hochbaum AI, Kolodkin-Gal I, Foulston L, Kolter R, Aizenberg J, Losick R.**
408 2011. Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm
409 development. *J Bacteriol* **193**:5616-5622.
- 410 37. **Lark C, Lark KG.** 1961. Studies on the mechanism by which D-amino acids block
411 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, Alghafari 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 RIG, 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.

- 483 62. **Scheffe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H.** 2006.
484 Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene
485 expression’s C T difference” formula. *J Mol Med* **84**:901-910.
- 486 63. **Peeters E, Nelis HJ, Coenye T.** 2008. Comparison of multiple methods for
487 quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods*
488 **72**:157-165.
- 489 64. **Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK,**
490 **Roy R, Singh S.** 2005. Mechanism of thioflavin T binding to amyloid fibrils. *J Struct*
491 *Biol* **151**:229-238.
- 492 65. **Biancalana M, Koide S.** 2010. Molecular mechanism of Thioflavin-T binding to
493 amyloid fibrils. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*
494 **1804**:1405-1412.
- 495
- 496