Growth rates and Metabolic Traits Differentiate *Campylobacter jejuni* Strains by Diarrheal Manifestation

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Short title: Metabolism and growth differences drive *Campylobacter jejuni* diarrheal manifestations

Abstract

Campylobacter jejuni is the leading bacterial cause of gastroenteritis worldwide, and can result in two different diarrheal manifestations in humans: a bloody/inflammatory diarrhea or a watery diarrhea. Currently, little is known about the factors leading to these two distinct diarrheal manifestations; however, five strains of *C. jejuni* associated with bloody/inflammatory diarrhea and five strains associated with watery diarrhea have been characterized in the neonatal piglet diarrhea model system. Here, we demonstrate individual differences between *C. jejuni* strains and found that these two groups of *C. jejuni* strains exhibit differences in temperature dependent and independent growth rate and carbon source utilization. We show that that watery diarrhea associated *C. jejuni* strains grow significantly faster and have enhanced biofilm production compared to bloody/inflammatory diarrhea associated *C. jejuni* strains at human body temperature (37ºC). Whereas bloody/inflammatory diarrhea associated strains grew faster at 42ºC compared to 37ºC. Using phenotypic microarrays to examine 192 carbon source utilization as a preliminary screening, we show that L-fucose is utilized differently by strains within the two diarrheal manifestation groups. L-fucose utilization is significantly higher among watery diarrhea associated strains at both 37ºC and 42ºC compared to bloody/inflammatory associated strains. While L-glutamine utilization is significantly higher among bloody/inflammatory diarrhea associated strains at 37ºC compared to watery associated strains. Overall, these findings suggest that there are distinct metabolic adaptations of bloody/inflammatory and watery diarrhea-associated *C. jejuni* strains to human (37ºC) and poultry (42ºC) temperatures that may contribute to their differing diarrheal manifestations.

46 **Introduction**

47 *Campylobacter jejuni* is a major cause of bacterial gastroenteritis, and is responsible for an
48 estimated 550 million cases per year worldwide[1]. Patients with campylobacteriosis caused by *C.*
49 *jejuni* typically experience symptoms within 2-5 days after exposure, which can include abdominal
50 cramping, nausea, and fever along with diarrhea that can be watery or bloody/inflammatory[2-4].
51 The diarrheal manifestations of *C. jejuni*, watery and/or bloody/inflammatory, have been noted for
52 several decades[5; 6], yet little has been explored concerning the role of *C. jejuni* phenotypic
53 characteristics in these outcomes. Watery diarrhea is generally characterized by loose non-bloody
54 stools, while the more severe bloody/inflammatory diarrhea includes the presence of blood in the
55 stool and/or inflammation of the ileum or colon[4; 7]. Campylobacteriosis is associated with
56 several post-infectious sequelae including Guillain-Barré syndrome and post-infectious irritable
57 bowel syndrome (PI-IBS)[8; 9]. Previous studies have suggested that severe, bloody diarrhea is
58 associated with increased invasiveness[10-12] and cytotoxin production[13-15], while watery
59 diarrhea has been linked to secretory mechanisms and enterotoxin activity[16-20]. However,
60 fundamental bacterial traits such as growth rate, carbon source utilization, motility, and biofilm
61 formation, key factors in colonization and persistence, have not been systematically examined in
62 the context of diarrheal manifestation.

63 *C. jejuni* isolates are phenotypically diverse including in their LOS[21; 22],
64 biofilm/aerotolerance[23], antimicrobial resistance, in their ability to produce PI-IBS[24], immune
65 response[25], phase-variation[26] or within a single sequence type (ST)[27; 28], among agriculture
66 strains[29] and even laboratory strains demonstrate considerable variability following multiple
67 growth passages[30-34]. Unlike many enteric bacteria, *C. jejuni* generally lacks key pathways
68 necessary for the metabolism of most carbohydrates in the intestinal tract such as glucose and

69 galactose[35]. *C. jejuni* has a relatively small genome and few identified metabolic pathways
70 compared to other enteric pathogens, such as *Salmonella,* but despite this, it has unique metabolic
71 flexibility and can utilize substrates such as amino acids or citric acid cycle intermediates[31; 36;
72 37]. Metabolic heterogeneity has been observed between *C. jejuni* strains with some strains able
73 to metabolize glutamine and asparagine[38]. In fact, the amino acid L-glutamine can be utilized as
74 a primary energy source in *C. jejuni* strains containing γ-glutamyl transpeptidase (GGT)[39].
75 Strains of *C. jejuni* that contain GGT, such as the sequence type 45 clonal complex (ST-45 CC)[40]
76 have been shown to have a colonization advantage in murine models. However, other *C. jejuni*
77 strains may be able to compensate for the lack of GGT by utilizing other metabolic pathways[38;
78 41]. Additionally, certain *C. jejuni* strains, particularly those of ST-22 CC[40], can contain a
79 genomic island for L-fucose metabolism, a sugar present in mucin glycoproteins in the intestinal
80 tract[42; 43]. L-fucose metabolism provides a competitive advantage during colonization of
81 certain hosts, however, its role in human pathogenesis remains unclear[42; 44]. Metabolic diversity
82 among *C. jejuni* strains may have a role in colonization and virulence differences within different
83 hosts, including strain variation of diarrheal manifestations in humans. Colonization factors like
84 growth, motility, biofilm production, and carbon utilization have rarely been studied for their
85 potential role in the diarrheal manifestation of *C. jejuni* yet understanding them is crucial to
86 furthering understanding the pathogenesis of *C. jejuni*.
87 Here, we investigated whether growth characteristics and metabolic capacity differ between strains
88 of *C. jejuni* associated with distinct diarrheal manifestations, and whether these differences are
89 consistent with host adaptation or zoonotic potential. Using a panel of ten well-characterized *C.*
90 *jejuni* strains shown to cause either watery or bloody/inflammatory diarrhea in the neonatal piglet
91 model - a system that reliably recapitulates human disease phenotypes[45; 46], we compared

92 generation times, carbon source utilization (L-fucose, L-glutamine, and mucin), motility, and
93 biofilm formation across temperatures representative of human (37°C) and avian (42°C) hosts.
94

95 **Materials and Methods**

96 **Bacterial Culturing and Growth**

97 Strains used in this study, their source and associated diarrheal manifestation in the neonatal piglet
98 model are listed in **Table 1**. The strains selected for this study were chosen based on their
99 documented diarrheal manifestations in neonatal piglets, offering a more controlled way to
100 investigate the mechanisms behind the observed clinical variability. All watery diarrhea associated
101 strains were sourced from poultry, while two strains associated with bloody/inflammatory diarrhea
102 originated from poultry and the other three were isolated from human clinical cases. In addition to
103 the *in vivo* work that has been performed with these strains, all have been characterized either
104 genotypically or phenotypically to varying degrees in previous studies, with strains NCTC11168,
105 81-176 and M129 the most well characterized. However, none of these strains have been
106 investigated as part of two diarrheal manifestation groups for their growth and metabolic
107 capabilities as has been done in this study. All *C. jejuni* strains were grown and maintained on
108 Mueller-Hinton agar plates supplemented with 5% defibrinated bovine blood (MHB; Becton,
109 Dickinson and Company Difco) for 48 hours at 37°C with 10% CO_2. Strains for assays that
110 required liquid culture were grown in Mueller-Hinton (MH; Becton, Dickinson and Company
111 Difco) broth with constant shaking (100 rpm) for 16-18 hours at 37°C with 10% CO_2.

112

113 **Growth Curves**

114 Growth curves in MH broth were conducted for each of the *C. jejuni* strains. Overnight cultures
115 were grown with shaking (100 rpm) at 37°C with 10% CO_2, OD_{600} measured, and then diluted to
116 a final OD_{600} of 0.001 (~1 x 10^6 CFU/ml) with MH broth. Next, 300 µl aliquots of the diluted
117 inoculum were added in triplicate to wells in a sterile 96-well polystyrene plate (Fisher Scientific),
118 and the plate incubated at 37°C with 10% CO_2 for 1 hour before being sealed tightly with an optical
119 plate film cover (Applied Biosystems) to prevent evaporation during incubation. The sealed plate
120 was then placed in a SpectraMax M2 plate reader at either 37°C or 42°C, to investigate body
121 temperature differences between humans (37°C) and poultry (42°C; the primary *C. jejuni*
122 reservoir), with continuous shaking (100 rpm) for 36 hours with OD_{600} readings recorded every 15
123 minutes. Generation times for each strain were calculated using readings from the logarithmic
124 phase of growth. Three biological replicates were performed for all 10 *C. jejuni* strains with two
125 technical replicates (n = 3).
126
127 **Carbon Utilization Microarray**
128 Phenotype carbon utilization microarrays were conducted as described previously by Line et al
129 [47]. Briefly, phenotype microarrays (PMs) were conducted using the PM1 and PM2A plates
130 (Biolog Inc., CA, USA) for variation in carbon utilization sources between strains. Each strain of
131 *C. jejuni* was grown on MHB agar for 12 – 16 hours at 37°C with 10% CO_2. The bacteria were
132 collected from the MHB agar plate using an inoculating loop, resuspended in 17 ml of IF-0a
133 medium (Biolog Inc., CA, USA), and then diluted to a final OD_{600} of 0.8 in IF-0a medium. Next,
134 the inoculum suspension was mixed with IF-0a, a phenotype microarray additive solution
135 containing tetrazolium violet (Redox dye D), according to manufacturer's instructions. Then 100
136 µl of the inoculum suspension was added to each well of either the PM1 or PM2A plates. The

137 plates were incubated at 37°C with 10% CO_2 for 48 hours before taking OD_{550} measurements in a
138 microplate reader (SpectraMax M2) and visually recording a color change from clear to violet in
139 each well on a scale of 0-3. A negative control plate for both types of plates was also incubated in
140 the absence of bacteria to determine false positives. Each strain was screened a single time with
141 both the PM1 and PM2A carbon utilization plates to serve as an initial screening method to indicate
142 potential carbon sources of interest to study further for the strains as described below (n = 1).
143
144 **L- Fucose, L- Glutamine, and Mucin Utilization Assays**
145 The phenotype microarray initial screening results indicated carbon utilization differences between
146 the diarrheal manifestation groups for both L-fucose and L-glutamine, thus we focused on these
147 carbon sources for additional studies. Studies on L-fucose and L-glutamine were done at both 37°C
148 and 42°C. Mucin utilization at 37°C was added as an additional assay as it has been shown to be
149 important to *Campylobacter* pathogenesis and L-fucose is a component of mucus. L-fucose, L-
150 glutamine, and mucin utilization assays were adapted from Stahl et al [42]. Briefly, overnight MH
151 broth cultures of the different *C. jejuni* strains were grown with shaking (100 rpm) then diluted to
152 a final OD_{600} of 0.01 with minimum essential medium (MEM; Gibco) without glutamine or phenol
153 red and supplemented with 20 μM $FeSO_4$. The media was also supplemented with either no
154 supplement, 25 mM L-fucose (Thermo Scientific), 0.01 mg/ml bovine submaxillary gland mucin
155 (Millipore) or 20 mM L-glutamine (Alfa Aesar) depending on the assay. Next, 300 μl aliquots of
156 the diluted inoculum with or without the appropriate supplement were inoculated in triplicate into
157 a sterile 96-well plate, incubated at 37°C with 10% CO_2 for 1 hour, then sealed tightly with an
158 optical plate film cover (Applied Biosystems) to prevent evaporation during incubation. The sealed
159 plate was then placed in a SpectraMax M2 plate reader at either 37°C or 42°C (for L-fucose and

160 L-glutamine) with continuous shaking (100 rpm) for 36 hours with OD_{600} reading recorded every
161 15 minutes. The plate included a blank control well containing 300 µl of uninoculated medium.
162 Results were recorded as the maximum OD_{600} obtained for the culture during 36 hours of growth
163 minus the blank media OD_{600}[39]. Each strain was tested in three biological replicates with three
164 technical replicates for each of the different carbon utilization assays with either L-fucose, mucin
165 or L-glutamine or without supplements (n = 3). There was no growth without any L-fucose, mucin
166 or L-glutamine present during any of the assays.
167
168 **Motility Assay (Soft Agar Swarming)**
169 Motility assays were conducted based on assays described previously by Reuter et al [48] with the
170 following modifications: (1) using an OD_{600} of 1.0; (2) checking at time points at 24 hours and 48
171 hours; (3) using a 10 µl inoculation into the agar; and (4) incubating the agar plates at either 37°C
172 or 42°C. Briefly, each *C. jejuni* strain was grown overnight in MH broth, the OD_{600} measured, and
173 the culture diluted in MH broth to a final OD_{600} of 1.0. Mueller Hinton (MH) soft agar plates
174 (Mueller Hinton broth supplemented with 0.4% agar) were inoculated with 10 µl of the diluted
175 inoculum directly into the center of the plate by stabbing into the agar. Inoculated plates were
176 incubated at either 37°C or 42°C with 10% CO_2 for 48 hours. The radius of growth was measured
177 after 24 hours and 48 hours of incubation using a digital calibrator starting from the edge of the
178 inoculum spot to the edge of growth. Each strain was inoculated onto two separate plates for two
179 technical replicates and each assay was conducted in three biologically independent assays (n =
180 3).
181
182 **Biofilm Assay**

183 Biofilm assays were performed as previously described by Reeser et al[49] except for the
184 following modification: (1) strains were inoculated at an OD_{600} of 0.25. Briefly, *C. jejuni* strains
185 were grown overnight in MH broth with shaking (100 rpm) at 37°C with 10% CO_2, the OD_{600} was
186 measured, and the culture diluted with MH broth to a final OD_{600} of 0.25. Then 100 µl of diluted
187 inoculum were then added to the corresponding well of a sterile 24-well polystyrene plate (Fisher
188 Scientific, Los Angeles, CA, USA) containing 1 ml of MH broth. The plate was then statically
189 incubated at 37°C with 10% CO_2 for 72 hours. After 72 hours incubation, all liquid media was
190 carefully removed from each well by pipetting to avoid disrupting the pellicle, and then the plate
191 was dried at 55°C for 30 minutes. The pellicle in each well was then stained by adding 1 ml of
192 0.1% crystal violet (100 mg of crystal violet powder (Acros Organics), 8 ml of sterile double
193 distilled water (ddH_2O), and 2 ml 100% methanol (Fisher Bioreagents)) and incubating at room
194 temperature for 5 minutes. Next, each well was washed twice with 1 ml of sterile ddH_2O to remove
195 any excess crystal violet, the plate was dried at 55°C for 15 minutes, and then decolorized by
196 adding 1 ml of decolorizer solution (80% ethanol (Fisher Bioreagents) and 20% acetone solution
197 (Fisher Bioreagents)). Finally, 100 µl of the decolorized solution for each strain was transferred to
198 a corresponding well of a 96-well polystyrene plate, and the absorbance at 570 nm was measured
199 using a SpectraMax M2 microplate reader. Each strain was tested in two technical replicates during
200 three biologically independent assays (n = 3).
201
202 **Statistical Analysis**
203 Statistical analyses were performed using GraphPad Prism (v10.4.1) for comparisons between
204 diarrheal manifestations or RStudio (v2022.07.1) for comparisons between strains. All data were
205 tested for normality (Shapiro–Wilk test) and equal variance (Levene's test). For comparisons

206 between strains associated with watery and bloody/inflammatory diarrhea (n = 15 per group), the
207 non-parametric Mann–Whitney U test was applied. Statistical significance was defined as $p \leq 0.05$.
208 For comparisons across multiple strains or conditions, non-parametric Kruskal–Wallis tests with
209 Dunn's post hoc test were used. Compact Letter Display (CLD) notation was used to visualize the
210 results of the pairwise comparisons. Strains that do not share a letter are significantly different
211 from each other ($p < 0.05$), while strains sharing the same letter are not significantly different.
212 Letters were assigned using the multcompView package in R, based on adjusted p-values.
213 Statistical significance was defined as $p \leq 0.05$. Each assay included a minimum of three
214 independent biological replicates per strain (n = 3), with technical replicates as noted. Data are
215 presented as mean ± SEM unless otherwise stated.
216
217 **Results**
218 ***Watery diarrhea-associated C. jejuni strains grow faster than bloody/inflammatory strains at***
219 ***37°C***
220 To evaluate whether differences in growth dynamics could explain divergent clinical
221 manifestations, we compared the growth kinetics of five watery diarrhea-associated and five
222 bloody/inflammatory-associated *C. jejuni* strains at two host-relevant temperatures: 37°C (human)
223 and 42°C (poultry). Growth was monitored over 36 hours using OD_{600} measurements taken every
224 15 minutes. Strain to strain differences were observed widely among both 37°C and 42°C, and
225 strains D42a, M129, D33a, A9a and NCTC11168 all grew significantly slower at 37°C than at
226 42°C ($p < 0.05$; **Figure 1A**).
227 At both temperatures, watery diarrhea associated strains entered logarithmic growth phase earlier
228 and reached higher OD_{600} values compared to bloody/inflammatory strains (**Supplementary**

229 **Figure 1A–D**). One watery strain (D42a) displayed a slower growth profile, similar to that of
230 bloody/inflammatory strains. Generation times calculated from logarithmic growth phase found
231 significantly faster generation times for watery diarrhea associated strains compared to
232 bloody/inflammatory associated strains at 37°C but not at 42°C (37°C watery diarrhea associated
233 115.8 minutes, 37°C bloody/inflammatory diarrhea associated 139.99 minutes $p = 0.018$; 42°C
234 watery diarrhea associated 95.23 minutes, bloody/inflammatory diarrhea associated 42°C 111.47
235 minutes $p = 0.38$; **Figure 1B**). There was no significant difference in generation times at 37°C
236 compared to 42°C for watery diarrhea associated strains ($p = 0.12$) but bloody/inflammatory
237 diarrhea associated strains grew significantly faster at 42°C than 37°C ($p = 0.0057$).
238
239 ***Strains differ in utilization of L-fucose and L-glutamine but not mucin***
240 To determine whether nutrient utilization profiles differ between strains associated with distinct
241 diarrheal outcomes, we screened all ten *C. jejuni* strains for their ability to metabolize 192 carbon
242 sources using BIOLOG PM1 and PM2A phenotype microarray plates. There were a few carbon
243 sources used solely by one or two bloody/inflammatory diarrhea associated strains and not watery
244 associated strains and vice versa, including D-tagatose (D33a), succinnamic acid (D33a, 81-176),
245 citric acid (NCTC11168) and tricatballyic Acid (S3). Several carbon sources exhibited distinct
246 utilization patterns between the two diarrheal manifestation groups (**Supplementary Figure 2**).
247 L-serine was utilized by only three bloody/inflammatory diarrhea associated strains (M129, 81-
248 176 and NCTC11168) and no watery diarrhea associated strains, while L-glycyl-proline was
249 utilized by watery diarrhea associated strains A13a, A14a, S3 but only bloody/inflammatory
250 diarrhea associated strain A9a. Notably, L-glutamine and L-fucose were utilized by multiple
251 strains in only one group: L-glutamine was used by three bloody/inflammatory strains (81-176,

252 M129, D33a) and only one watery strain (S3), while L-fucose was used by four watery strains
253 (A13a, A14a, D42a, S3) and just one bloody/inflammatory strain (A9a). L-fucose and L-glutamine
254 were selected for further phenotypic analysis, along with mucin due to its relevance in host
255 colonization[50-52].
256 All five watery strains showed robust growth in the presence of L-fucose, with strain S3 reaching
257 the highest OD_{600} at 42°C (**Figure 2A**). At 37°C, the mean OD_{600} for watery strains was 0.077
258 compared to 0.035 for bloody/inflammatory strains ($p < 0.0001$); at 42°C, the difference was even
259 more pronounced (mean OD_{600}: 0.149 vs. 0.066, $p < 0.0001$). L-fucose supported significantly
260 greater growth in watery diarrhea-associated strains at both temperatures (**Figure 2B**). These
261 results indicate that L-fucose metabolism is a consistent and temperature-independent trait among
262 watery diarrhea-associated strains. To assess whether differences in mucosal substrate metabolism
263 extended beyond individual monosaccharides, we next tested the ability of each strain to grow in
264 medium supplemented with bovine submaxillary gland mucin at 37°C. Although there was strain-
265 level variability, no significant difference in mucin utilization was observed between watery and
266 bloody/inflammatory strains ($p = 0.67$), (**Figure 3, Supplementary Figure 3**).
267 L-glutamine utilization supported higher levels of growth among all strains compared to L-fucose,
268 with several significant differences between different strains at 37°C or 42°C (**Figure 4A**). Among
269 all strains tested, only A13a showed significantly higher growth at 42°C compared to 37°C, while
270 81-176 and A9a both showed increased growth at 37°C compared to 42°C. However, when
271 grouped by diarrheal manifestation, bloody/inflammatory associated strains exhibited enhanced
272 growth at 37°C in L-glutamine-supplemented medium compared to watery diarrhea associated
273 strains ($p = 0.0005$) (**Figure 4B**). There was no difference in growth potential at 42°C between
274 watery and bloody/inflammatory strains, ($p = 0.68$), suggesting a temperature or host dependent

275 role for glutamine utilization among *C. jejuni* strains. Together, these results demonstrate that
276 carbon source utilization differs consistently between strain groups and that these traits may be
277 modulated by host temperature. The enhanced L-fucose metabolism of watery diarrhea associated
278 strains may facilitate rapid growth and colonization in both poultry and human hosts, while the
279 slower growth and utilization of L-glutamine by bloody/inflammatory diarrhea associated strains
280 contributes to persistence within the human host.
281
282 ***Motility varies widely among C. jejuni strains but does not distinguish diarrheal phenotype***
283 Motility is critically important for *C. jejuni* colonization and pathogenesis, aiding in both adhesion
284 as well as invasion of host cells[53-55]. To assess whether motility differs between strains
285 associated with watery versus bloody/inflammatory diarrhea, we performed swarming motility
286 assays in soft agar at both 37°C and 42°C. A wide range of motility phenotypes was observed
287 among *C. jejuni* strains: bloody/inflammatory strains NCTC11168, D33a, and 81-176, and watery
288 strains D42a and RM1221, were highly motile (>10 mm radius), while bloody/inflammatory strain
289 A9a and watery strain S3 were weakly motile or non-motile (<10 mm) (**Figure 5**). While no
290 significant difference in motility was observed between the two diarrheal manifestations at either
291 temperature, a definite trend toward reduced motility among bloody/inflammatory diarrhea
292 associated strains was noted at 37°C ($p = 0.052$). No such trend was observed at 42°C, where the
293 difference remained non-significant ($p = 0.66$; **Supplementary Figure 4**). There was no
294 significant difference in motility at different temperatures for the bloody/inflammatory ($p = 0.18$)
295 or watery ($p = 0.073$) diarrhea associated strains, but watery diarrhea associated strains trended
296 towards significance.
297

298 ***Biofilm formation differs by diarrheal phenotype***

299 Biofilm formation contributes to the persistence, and transmission of *C. jejuni*[56]. Therefore, to
300 assess the potential differences in these traits among our *C. jejuni* strains, we quantified biofilm
301 formation at 37°C using crystal violet staining. Strong biofilm producers (A_{570} > 0.30) included
302 bloody/inflammatory diarrhea associated strain M129 and watery diarrhea associated strains A13a
303 and D42a. Medium biofilm formation (A_{570} between 0.20–0.30) was observed in
304 bloody/inflammatory diarrhea associated strains 81-176 and NCTC11168 and watery diarrhea
305 associated strains A14a and S3, while poor biofilm formation (A_{570} < 0.20) was noted for A9a,
306 D33a, and RM1221 (**Figure 6A**).Although strains varied in their individual capacities, biofilm
307 formation at 37°C was found to significantly differ between diarrheal manifestation groups (p =
308 0.019; **Figure 6B**).

309

310 **Discussion**

311 This study investigated the variation between strains of *C. jejuni* with defined diarrheal phenotypes
312 in the neonatal piglet model, we assessed growth rates, carbon source utilization, motility, and
313 biofilm formation at human and avian body temperatures. In human and poultry hosts, *C. jejuni*
314 must pass through the mucosal layer of the intestines to reach the intestinal epithelial surface and
315 establish an infection. Here, *C. jejuni* strains showed significant strain to strain variation, including
316 differences in the utilization of L-fucose, L-glutamine, generation times, and biofilm formation,
317 but did not display significantly different abilities to utilize bovine submaxillary gland mucin when
318 grouped by diarrheal manifestation, findings that were not surprising based on *C. jejuni's* limited
319 ability to metabolize carbohydrates[35]. It is known that *C. jejuni* growth is enhanced when co-
320 cultured with commensal *Bacteroides vulgatus*, a response suggested to be due to the breakdown

321 of mucin by *B. vulgatus*[57]. There are several types of bacteria that are known mucin degraders
322 that have been identified in humans, calves and goats, and include *Bifidobacterium*[58; 59],
323 *Bacteroides*[59] and *Akkermansia muciniphila*[60]. Mucin degradation by *A. muciniphila* is
324 known to support intestinal barrier function[61] and provide physiological benefits that prevent
325 disease[61-63]. The composition of mucin degraders in chickens is less well defined, but
326 *Bacteroides* has been found to be one of the most predominating genera of bacteria in the chicken
327 intestinal microbiome[62; 64]. The growth of these commensal bacteria is supported by the highly
328 glycosylated mucins within the intestinal mucus[65], during which active fructosidases are
329 secreted[66; 67]. *C. jejuni* lacks the ability to release L-fucose from glycans and instead is known
330 to utilize free fucose from mucin degraders such as *B. vulgatus*[57].
331 The mucus layer of the intestinal tract is where *C. jejuni* encounters L-fucose, the only known
332 carbohydrate chemoattractant for *C. jejuni*[68; 69]. In the human gut, 80% of the total dry weight
333 of intestinal mucins is made up of *O*-linked glycans[70; 71], however in poultry L-fucose is
334 suggested to comprise an estimated 7% of the *O*-glycans in the intestinal tract[72]. The *fuc* locus
335 for L-fucose catabolism has been identified in >50% of sequenced *C. jejuni* isolates[73] and has
336 been observed among a high percentage of *C. jejuni* isolates from humans[74]. Here we found
337 that strains associated with watery diarrhea exhibited enhanced utilization of L-fucose at 37°C and
338 also grew significantly faster than those linked to bloody/inflammatory disease at 37°C and 42°C.
339 We hypothesis that this combination of rapid growth and efficient metabolism may support early
340 colonization and transmission, contributing to the acute nature of watery diarrhea.
341 The concentration of L-fucose is particularly high among breastfed infants due to the secretion of
342 L-fucose from fucosylated human milk oligosaccharides by intestinal commensals such as
343 *Bifidobacterium* spp and *Bacteriodes* spp[57; 67; 75; 76]. The amount of available L- fucose is

344 known to be as high as 4-5 mg/g in the feces of breastfed infants and in low to middle income
345 countries, higher proportions of *C. jejuni* have been found among these infants compared to those
346 that were not breastfed[77; 78]. Despite the high levels of available L-fucose, breastfed infants
347 with campylobacteriosis had significantly higher proportions of non-L-fucose metabolizing
348 *Campylobacter* than non-breastfed infants[77], a finding suggested to be driven in part by L-fucose
349 chemotaxis[79].
350 Chemotaxis towards L-fucose in the intestinal lumen through the upregulation of L-fucose
351 dehydrogenase, FucX, is suggested to contribute to its clearance while non-L-fucose metabolizing
352 *C. jejuni* strains move towards the intestinal mucosa to establish disease[79]. These findings
353 support the hypothesis that watery diarrhea associated strains utilize L-fucose in the mucus layer
354 where they are maintained further away from the intestinal epithelial cells while
355 bloody/inflammatory strains move to establish themselves at the enterocyte surface. Indeed, we
356 have recently found that bloody/inflammatory diarrhea associated *C. jejuni* strains are significantly
357 more invasive than watery diarrhea associated strains (Bosquez et al 2025). However, in contrast,
358 L-fucose utilization has also been observed *in vitro* to enhance invasion of Caco-2 cells and
359 significantly increase fibronectin binding efficacy in strain NCTC11168[44]. Another study found
360 similar results with invasion of Caco-2 increased with the addition of 10 mM L-fucose under
361 microaerophilic conditions using *C. jejuni* strain 108[67]. The same study observed growth
362 enhancement of *C. jejuni* strain 108 in the presence of L-fucose, a finding similar to ours,
363 suggesting that watery diarrhea associated strains may alter their invasiveness if tested under the
364 same conditions. In addition, we found that the increased growth potential of watery diarrhea
365 associated strains was maintained at both host-relevant temperatures, suggesting adaptation to
366 colonization across multiple hosts with enhanced growth in humans. The ability to utilize L-fucose

367 provided a colonization advantage *in vivo* in piglets but had no effect on the colonization of
368 chickens when inoculated at a low dose[42]. Since piglets are a model for human disease, whereas
369 chickens are the natural reservoir for *C. jejuni*, this suggests L-fucose utilization at 37°C (human
370 body temperature) versus 42°C (chicken body temperature) may only be relevant to human
371 disease.

372 In contrast, bloody/inflammatory diarrhea-associated strains grew more slowly particularly at
373 37°C, a factor that potentially contributes to prolonged colonization and disease development.
374 Slower growth may allow for slower establishment of an infection, which allows for delayed
375 immune recognition of the pathogen until the bacteria more established, potentially resulting in a
376 more damaging infection. Initial BIOLOG screening found that some strains preferentially utilized
377 L-glutamine, we observed a significant increase in growth potential in the presence of L-glutamine
378 by bloody/inflammatory associated strains compared to watery diarrhea associated strains at 37°C
379 but not at 42°C. In humans, glutamine is known to support epithelial barrier function and modulate
380 host immune responses, and its depletion may promote tissue damage and inflammation[80-82].
381 Because L-glutamine is a key fuel source for enterocytes and supports tight junction integrity[80;
382 83], *C. jejuni* utilization of L-glutamine may not only provide a metabolic advantage but also
383 actively disrupt enterocyte homeostasis. In the context of inflammation, where glutamine demand
384 is heightened due to increased epithelial turnover and immune cell activation, its depletion could
385 contribute to impaired repair to tissue damage as well as reduced immune function[84]. Other
386 studies have found that *C. jejuni* strains have considerable variation in their ability to utilize L-
387 glutamine, with 31% of strains[40] containing the enzyme gamma-glutamyl transpepidase (GGT)
388 necessary for glutamine and glutathione metabolism. One study found a higher percentage of *C.*
389 *jejuni* strains from human isolates (37.93%) to contain the *ggt* gene compared to 14.29% among

390 strains from non-human sources[51], suggesting an advantage for these strains in the human host.
391 Furthermore, a study of 166 *C. jejuni* strains from Finnish patients found an association between
392 bloody diarrhea and GGT production[85], while another study found purified *C. jejuni* GGT
393 impaired intestinal cell and lymphocyte proliferation[86]. The complicated and variable response
394 by *C. jejuni* strains containing *ggt* suggests that individual strains may have complex metabolic
395 requirements or can compensate with alternative pathways not yet characterized. Overall, the
396 metabolic diversity observed among strains highlights the importance of considering host-specific
397 and strain-specific adaptations when investigating L-glutamine utilization among *C. jejuni* strains,
398 especially among those associated with bloody/inflammatory diarrhea.
399 This study found that motility, a critical factor associated with colonization/pathogenicity[87-90],
400 had no significant differences between the different diarrheal manifestation groups. In terms of
401 motility, *Campylobacter* species utilize a single or bipolar flagella to move[91]. Upon entering a
402 host intestinal tract, the ability to move is important for navigating the thick layer of mucus to
403 reach the epithelial surface and colonize[91]. In this study, motility was variable at the strain level
404 and did not correlate strongly with diarrheal manifestations at either 37°C or 42°C in this study.
405 All strains were motile except strain S3, which was originally able to colonize poultry[92; 93], but
406 minimal laboratory passages rendered it defective for colonization[94]. Previously, similar results
407 were demonstrated for nonmotile *C. jejuni* strain CS that caused few pathological changes in the
408 piglet model, but grew to a greater maximum cell density in MH medium compared to a
409 genotypically similar but motile strain[88]. Watery diarrhea associated strains with limited to no
410 motility, such as S3, may be able to overcome the mucus layer via the more rapid growth that we
411 observed in this study, but additional studies are needed to confirm this hypothesis. Our results

412 suggest that motility is essential for the pathogenesis of *C. jejuni* regardless of the diarrheal
413 manifestation associated with the strain.
414 Biofilm formation is known to differ among *C. jejuni* strains[95] and has been suggested to
415 promote survival, transmission and colonization of hosts[96; 97]. The present study revealed
416 significantly higher biofilm formation among watery diarrhea associated strains at 37°C,
417 suggesting that these strains may have a colonization advantage in humans. In addition to
418 temperature, biofilm formation by bacteria can be triggered by various environmental stressors
419 such as nutrient availability, varying oxygen tensions and osmotic changes[98-100], for example,
420 one study showed that *C. jejuni* biofilm formation was increased under low-oxygen tensions as
421 well as in minimal nutrient medium[101]. An enhanced capacity to form biofilms would be
422 particularly useful in surviving the harsh conditions of the intestinal tract such as stomach acid,
423 bile salts, low-oxygen tension, oxidative stress or even immune defenses. When nutrients are
424 available, such as L-fucose, it is known that biofilm formation is reduced, and growth is enhanced
425 *in vitro*[73]. Future studies may investigate the ability of *C. jejuni* strains associated with watery
426 diarrhea to form biofilms when L-fucose is provided in the medium, particularly given their
427 enhanced growth with L-fucose. Stronger biofilm formation by strains associated with watery
428 diarrhea may be connected to their enhanced growth rate, which in combination would allow these
429 strains to quickly colonize the intestinal tract and contribute to the rapid production of watery
430 diarrhea.
431 It is important to note that our study revealed substantial phenotypic variability within each
432 diarrheal manifestation group. This is not unexpected given the known genomic and functional
433 diversity of *C. jejuni*, but it reinforces the need to study multiple strains to identify group-level
434 trends. Within the watery diarrhea group, strain D42a had a longer generation time at 37°C than

435 the other watery associated strains. S3, RM1221 and A14a had higher growth potential at 42°C in
436 the presence of L-fucose, while A13a showed significantly higher growth at 42°C in the presence
437 of L-glutamine. Among bloody/inflammatory strains, NCTC11168, A9a, D33a and M129 all had
438 slower generation times at 37°C than at 42°C. 81-176, D33a and NCTC-11168 showed similar
439 levels of motility, but A9a and M129 were only weakly. The presence of these outliers highlights
440 the spectrum of metabolic and growth capabilities within each pathotype and suggests that these
441 diarrheal manifestations may require several phenotypes to be present and may also rely on host
442 factors.
443 Another consideration is that strain classification in this study was based on diarrheal outcomes
444 observed in the neonatal piglet model, rather than direct human clinical data. While this may limit
445 immediate generalizability, the piglet model is the only experimental systems that reliably
446 reproduces both watery and bloody/inflammatory diarrheal manifestations caused by *C. jejuni.* It
447 provides a controlled environment to directly associate specific bacterial traits with clinical
448 outcomes, eliminating many of the confounding variables inherent in human observational studies.
449 Additionally, clinical strains are likely biased towards bloody/inflammatory diarrhea associated
450 strains, as people suffering from the less severe watery diarrhea are not likely to seek medical
451 attention. Nonetheless, validation of these findings using well-characterized human isolates with
452 known clinical presentations will be important for confirming their broader relevance.
453 Despite these limitations, our findings provide evidence that clinical outcome in *C. jejuni* infection
454 may be shaped by fundamental differences in bacterial growth and metabolism. Strains associated
455 with bloody/inflammatory diarrhea are slower growers, utilizing L-glutamine for enhanced growth
456 at 37°C, while those linked to watery diarrhea appear optimized for rapid expansion, biofilm
457 formation, and mucus-derived sugar utilization. These strategies may reflect differing host

458 adaptation trajectories and suggest that metabolic profiling could provide new insight into the
459 pathogenic potential of *C. jejuni* lineages.
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468 **Contributors**

469 **JB:** Conceptualization, methodology, experimentation, formal analysis, writing – original draft,
470 and review & editing. **KKC:** Conceptualization, methodology, resources, funding acquisition,
471 project administration, supervision, writing, and review & editing. **CP:** Writing, and review &
472 editing. **BP:** Writing, and review & editing.

473

474 **Conflicts of Interest**

475 The authors have no conflicts of interest.

476

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492 **Table 1.** *Campylobacter jejuni* strains utilized in the study and associated diarrheal manifestation

Strain	Source	ST	Clonal Complex	Diarrheal Manifestation
NCTC11168	Clinical	ST43	ST-21	Bloody and/or Inflammatory
81-176	Clinical	ST604	ST-42	Bloody and/or Inflammatory
M129	Clinical	ST353	ST-353	Bloody and/or Inflammatory
A9a	Poultry	ST2827	ST-460	Bloody and/or Inflammatory
D33a	Poultry	ST459	ST-42	Bloody and/or Inflammatory
RM1221	Poultry	ST354	ST-354	Watery
S3	Poultry	ST354	ST-354	Watery
D42a	Poultry	ST21	ST-21	Watery
A14a	Poultry	ST1212	ST-607	Watery
A13a	Poultry	ST1212	ST-607	Watery

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504 **Figure Legends**

505 **Figure 1. Generation time of *C. jejuni* strains at 37°C and 42°C.** (A) Generation Time in
506 minutes at 37°C and 42°C for all *C. jejuni* strains. Average generation time calculated from
507 logarithmic phase from three biologically independent assays with three technical replicates at
508 both 37°C and 42°C (n = 3). Compact letter displays (CLDs) indicate statistically significant
509 differences between groups ($p < 0.05$) as determined by Kruskal-Wallis test followed by Dunn's
510 post hoc test. Bars labeled with different letters indicate statistically significant differences ($p <$
511 0.05) between those strains and/or temperatures, whereas shared letters denote groups that are not
512 significantly different from each other. (B) Generation time in minutes of *C. jejuni* strains grouped
513 by diarrheal manifestation (n = 15). There was a significant difference between
514 bloody/inflammatory and watery diarrhea associated strains at 37°C (*, $p = 0.018$) but not at 42°C
515 ($p = 0.38$). There was a significant difference between 37°C and 42°C among
516 Bloody/inflammatory diarrhea associated strains (**, $p = 0.0057$), but among watery diarrhea
517 associated strains ($p = 0.12$).

518

519 **Figure 2. Utilization of L-fucose at 37°C and 42°C.** (A) L-fucose utilization at 37°C and 42°C
520 between *C. jejuni* strains. Compact letter displays (CLDs) indicate statistically significant
521 differences between groups ($p < 0.05$) as determined by Kruskal-Wallis test followed by Dunn's
522 post hoc test (n = 3). Bars labeled with different letters indicate statistically significant difference
523 ($p < 0.05$) between those strains and/or temperatures, whereas shared letters denote groups that are
524 not significantly different from each other. (B) L-fucose utilization at 37°C and 42°C between *C.*
525 *jejuni* strains grouped by diarrheal manifestation. There was a significant difference between the
526 growth of bloody/inflammatory and watery diarrhea associated strains when grown in minimal

527 media containing L-fucose at both 37°C (****, $p < 0.0001$) and at 42°C (****, $p < 0.0001$). The
528 average highest OD_{600} was calculated from three biologically independent assays with three
529 technical replicates (n = 15).
530
531 **Figure 3. Utilization of mucin at 37°C.** Bovine submucosally gland mucin utilization at 37°C by
532 *C. jejuni* strains. Compact letter displays (CLDs) indicate statistically significant differences
533 between groups ($p < 0.05$) as determined by Kruskal-Wallis test followed by Dunn's post hoc test.
534 Bars labeled with different letters indicate statistically significant difference ($p < 0.05$) between
535 those strains, whereas shared letters denote groups that are not significantly different from each
536 other. The average highest OD_{600} was calculated from three biologically independent assays with
537 three technical replicates (n = 3).
538
539 **Figure 4. Utilization of L-glutamine at 37°C and 42°C.** (A) Average highest OD_{600} L-glutamine
540 utilization at 37°C and 42°C across all *C. jejuni* strains. Compact letter displays (CLDs) indicate
541 statistically significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis
542 test followed by Dunn's post hoc test (n = 3). Bars labeled with different letters indicate statistically
543 significant difference ($p < 0.05$) between those strains and/or temperatures, whereas shared letters
544 denote groups that are not significantly different from each other. (B) Average highest OD_{600} L-
545 glutamine utilization at 37°C and 42°C across all *C. jejuni* strains grouped by diarrheal
546 manifestation. There was a significant difference between the growth of bloody/inflammatory and
547 watery diarrhea associated strains when grown at 37°C in minimal media containing L- glutamine
548 (***, $p = 0.0005$) but not at 42°C ($p = 0.68$). The average highest OD_{600} was calculated from three
549 biologically independent assays with three technical replicates (n = 15).

550 **Figure 5. Motility at 37°C and 42°C.** Average motility of *C. jejuni* strains after 48-hour
551 incubation at both 37°C and 42°C. Motility was measured as the radius of the halo of growth in
552 millimeters after 48-hour incubation. Average motility was calculated from three biologically
553 independent assays with two technical replicates (n = 3). Compact letter displays (CLDs) indicate
554 statistically significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis
555 test followed by Dunn's post hoc test (n = 3). Bars labeled with different letters indicate statistically
556 significant difference ($p < 0.05$) between those strains and/or temperatures, whereas shared letters
557 denote groups that are not significantly different from each other.
558
559 **Figure 6. Biofilm production at 37°C.** (A) Formation of biofilm after 72 hours at 37°C among
560 *C. jejuni* strains. Biofilm formation was quantified using spectrometer readings at 570 nm
561 following a 72-hour incubation at 37°C. Compact letter displays (CLDs) indicate statistically
562 significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis test followed
563 by Dunn's post hoc test. Bars labeled with different letters indicate statistically significant
564 difference ($p < 0.05$) between those strains, whereas shared letters denote groups that are not
565 significantly different from each other. Average biofilm formation was determined from three
566 biologically independent assays at 37°C with two technical replicates (n = 3) (B) Average biofilm
567 production of bloody/inflammatory and watery diarrhea associated strains after 72 hours at 37°C.
568 There was a significant difference between the biofilm production of watery and
569 bloody/inflammatory strains at 37°C (**, $p = 0.019$). Average biofilm production at 37°C was
570 calculated from three biologically independent assays with two technical replicates (n = 15).
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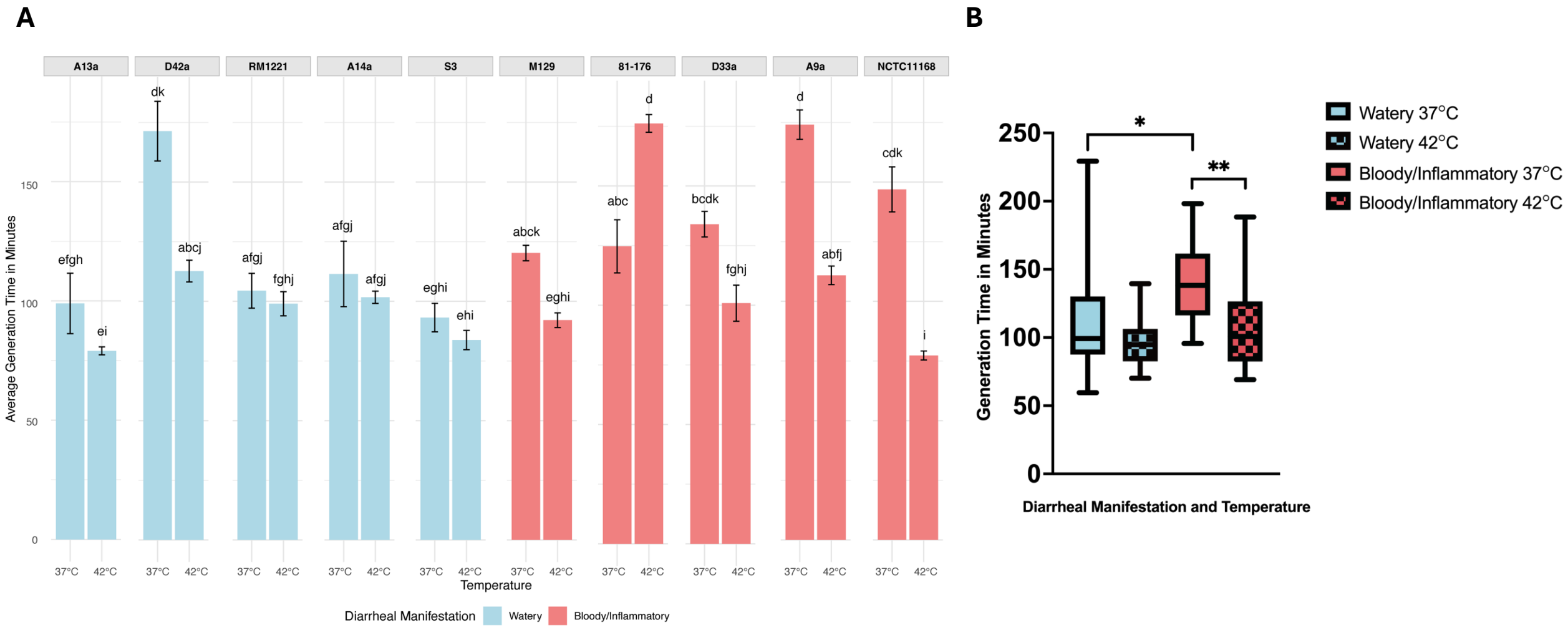
Figure 1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81-176
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100
150
efgh
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dk
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afgj
fghj
afgj
afgj
eghi
ehi
abck
eghi
abc
d
bcdk
fghj
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abfj
cdk
i
37°C
42°C
Temperature
Diarrheal Manifestation
Watery
Bloody/Inflammatory
B
Generation Time in Minutes
0
50
100
150
200
250
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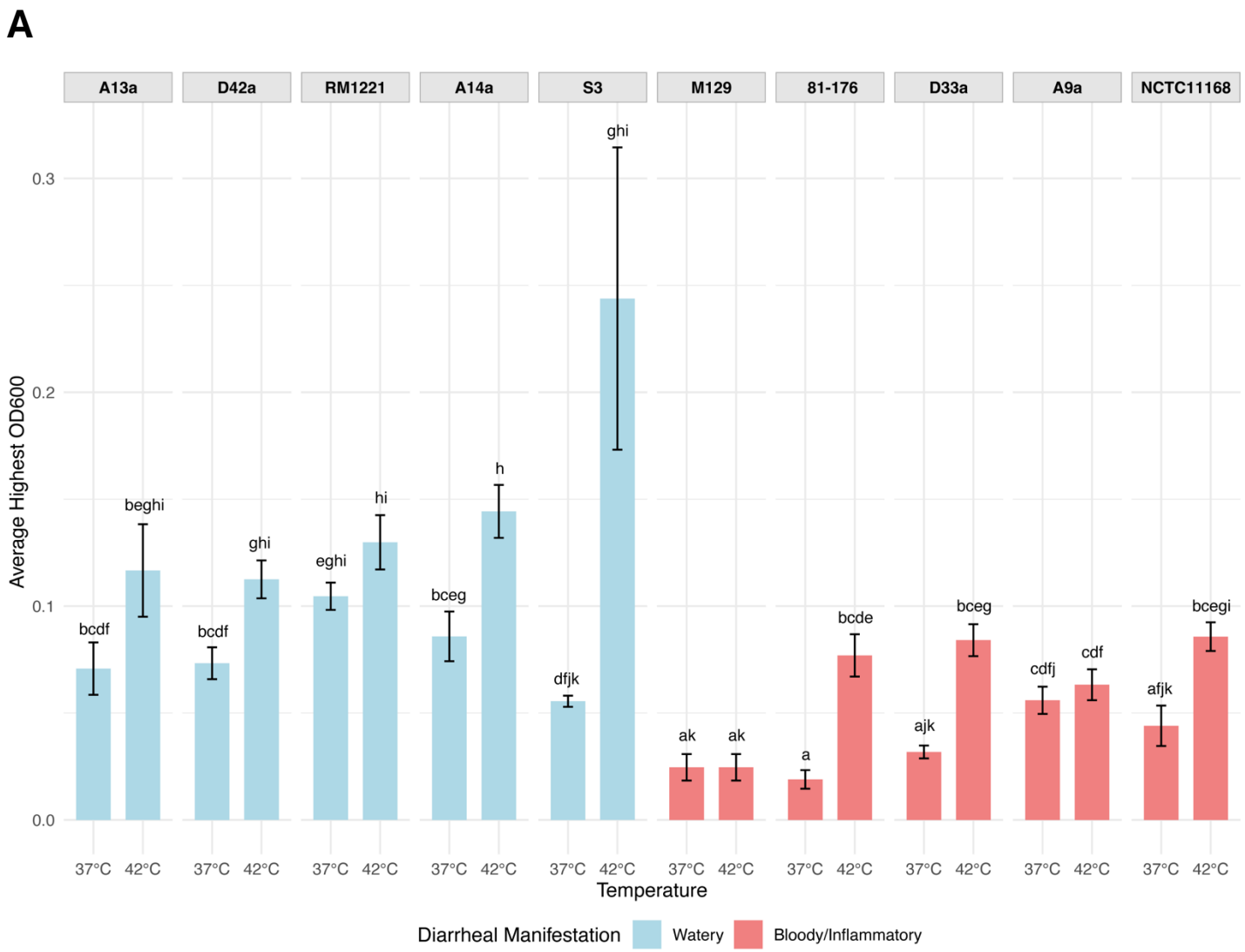
Diarrheal Manifestation and Temperature
Watery 37°C
Watery 42°C
Bloody/Inflammatory 37°C
Bloody/Inflammatory 42°C

Figure 2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hi
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a
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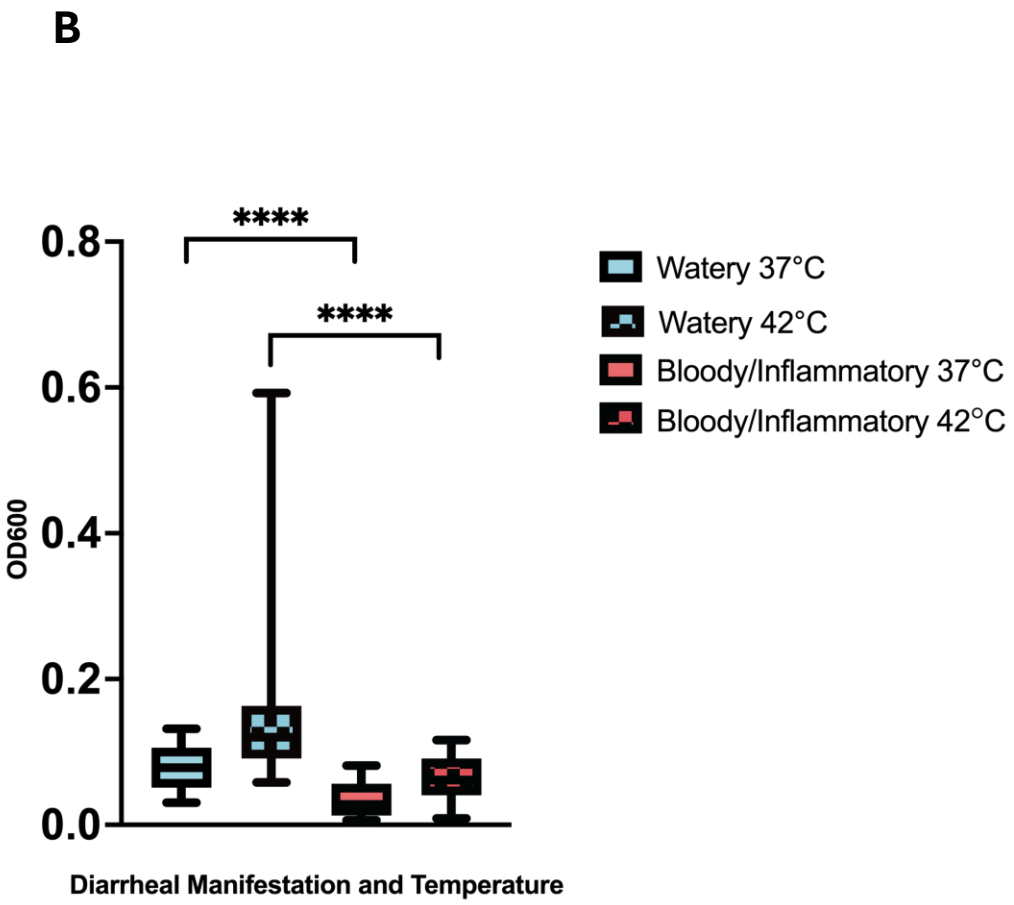
Temperature
Diarrheal Manifestation
Watery
Bloody/Inflammatory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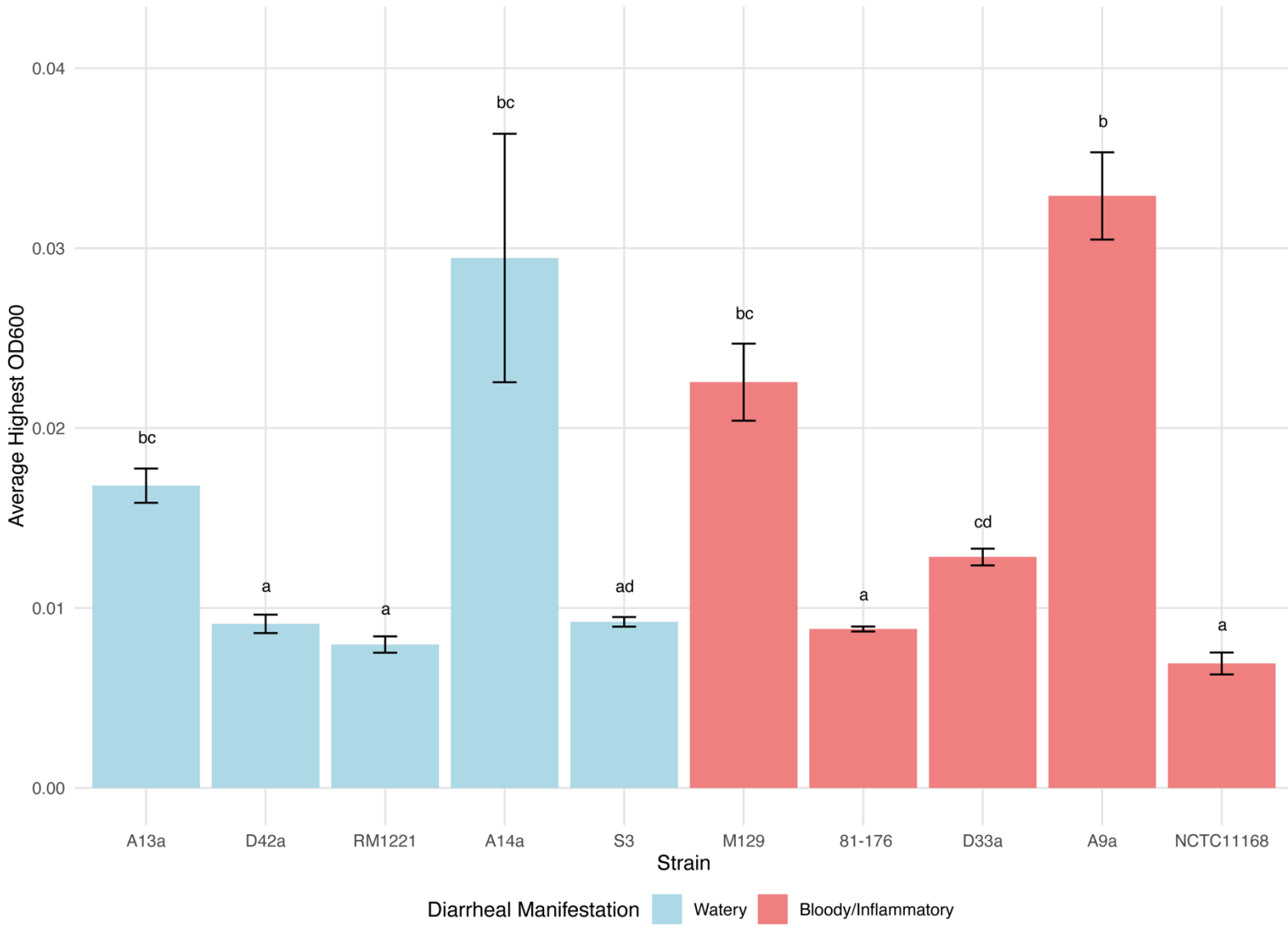
Watery 42°C
Bloody/Inflammatory 37°C
Bloody/Inflammatory 42°C

Figure 3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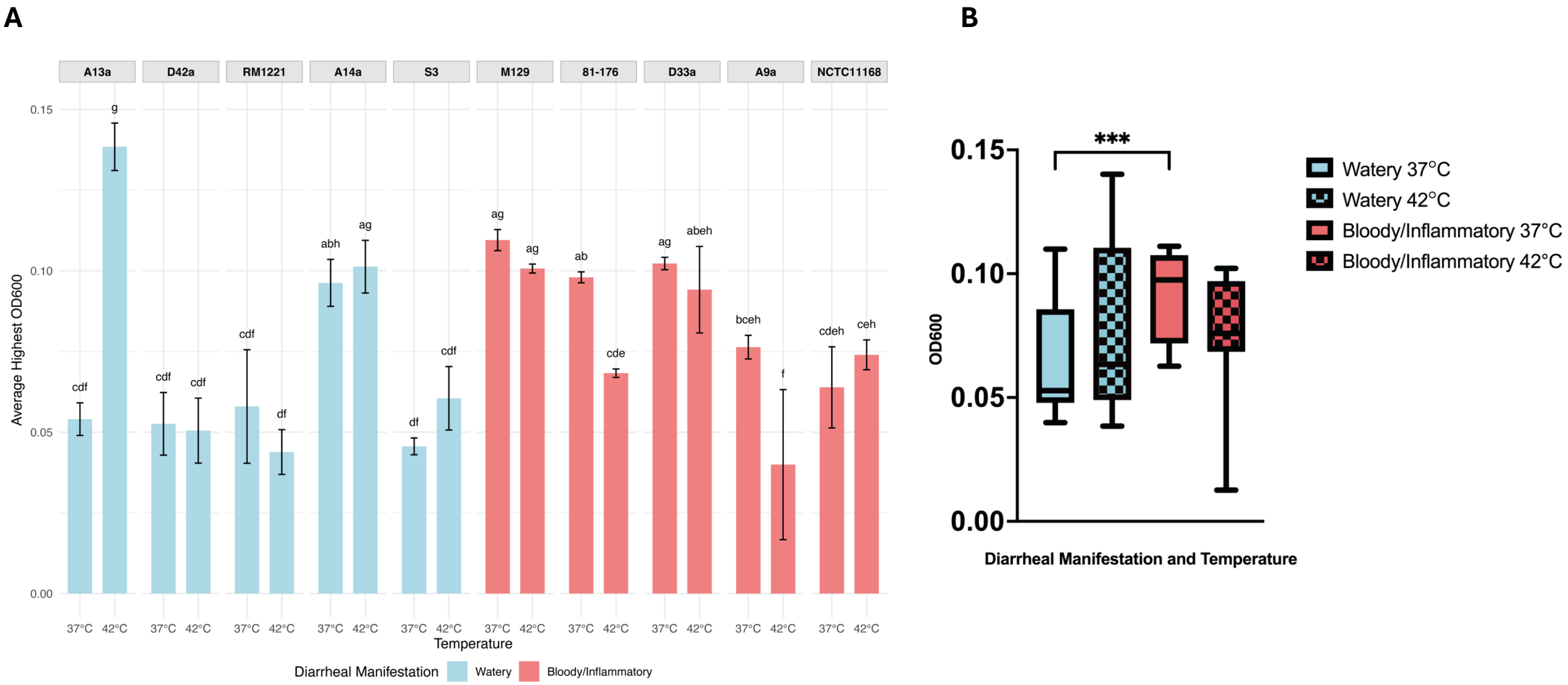
Strain
Diarrheal Manifestation
Watery
Bloody/Inflammatory

Figure 4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abeh
bceh
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cdeh
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37°C
42°C
Temperature
Diarrheal Manifestation
Watery
Bloody/Inflammatory
B
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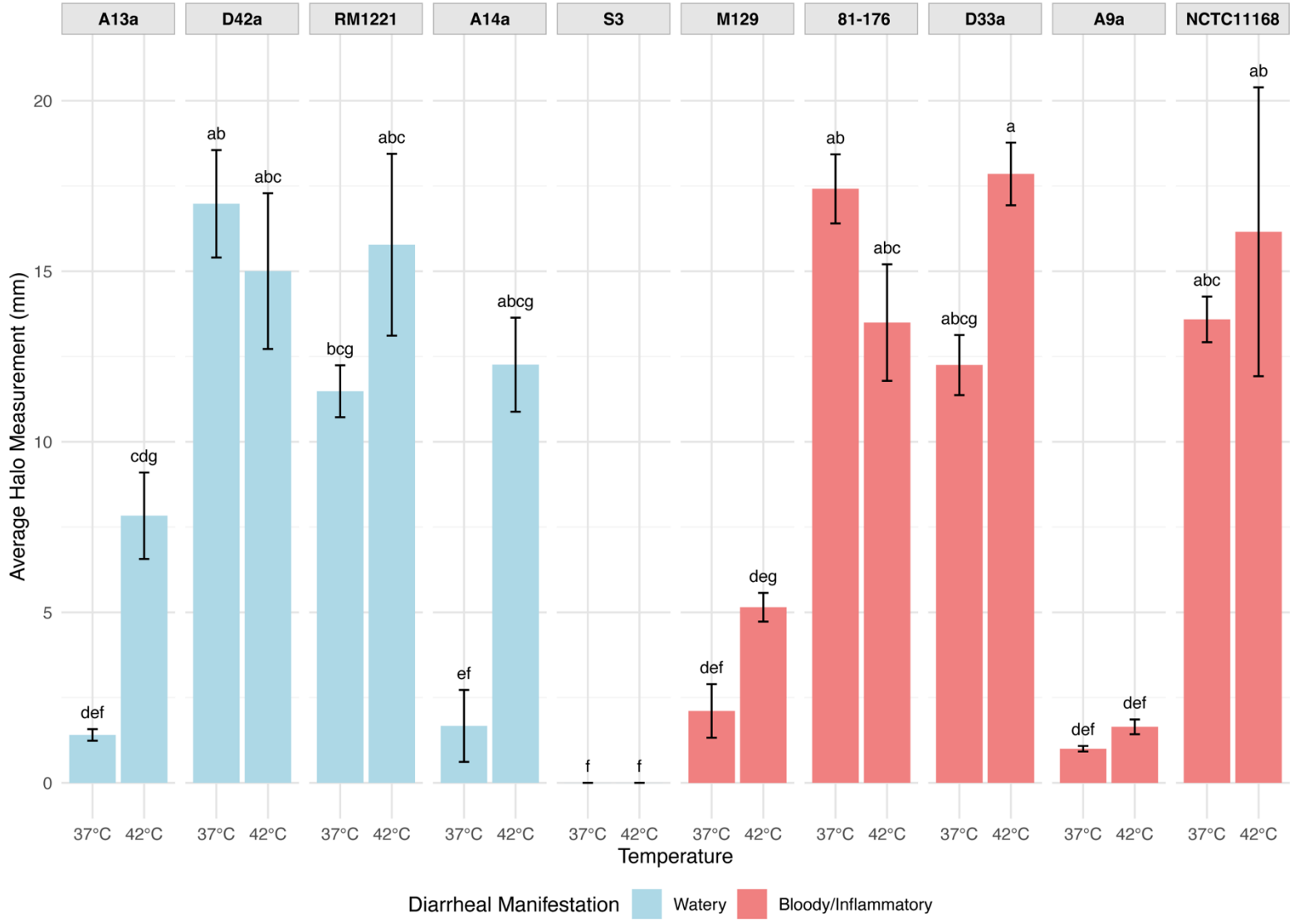
Watery 42°C
Bloody/Inflammatory 37°C
Bloody/Inflammatory 42°C

Figure 5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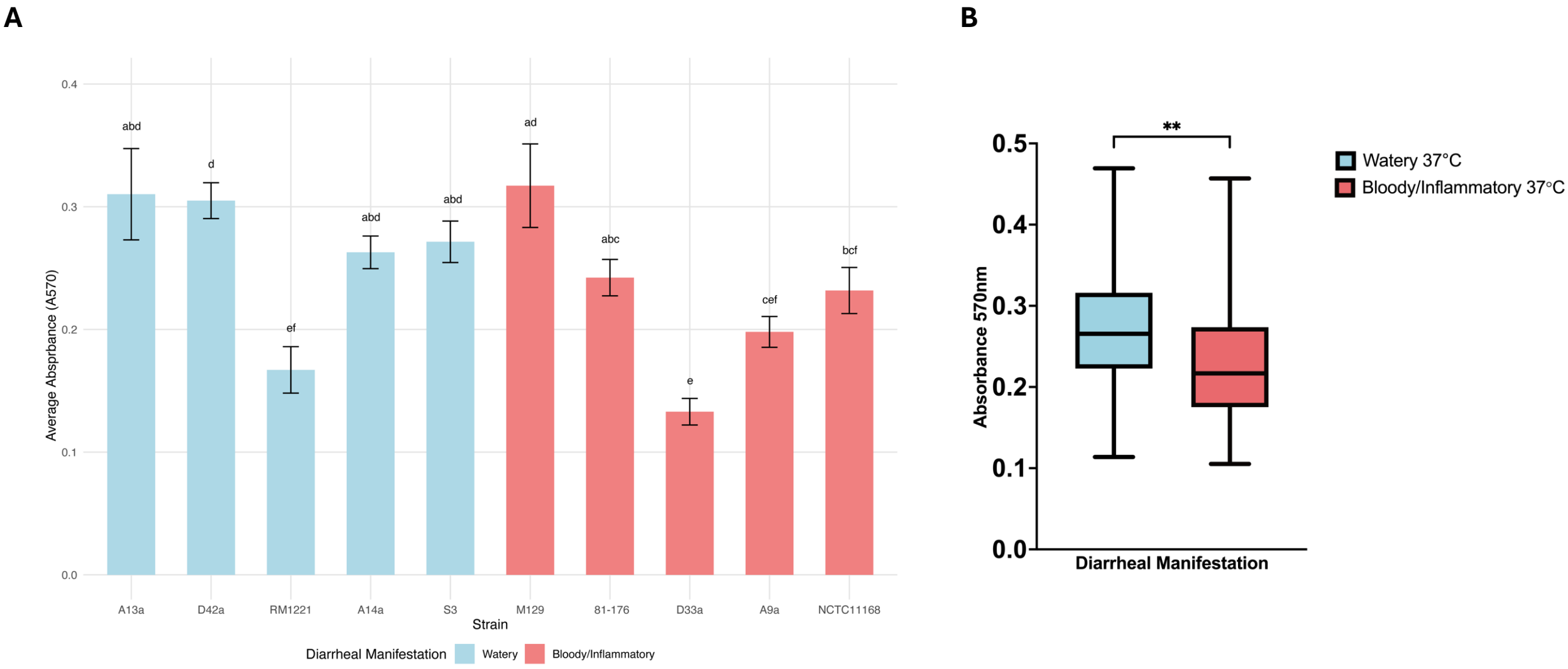
37°C
42°C
Temperature
Diarrheal Manifestation
Watery
Bloody/Inflammatory

Figure 6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bcf
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81-176
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NCTC11168
Strain
Diarrheal Manifestation
Watery
Bloody/Inflammatory
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0.4
0.3
0.2
0.1
0.0
Absorbance 570nm
Diarrheal Manifestation
Watery 37°C
Bloody/Inflammatory 37°C