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 rates and carbon source utilization. We show that watery diarrhea associated *C. jejuni* strains grow significantly faster compared to bloody/inflammatory diarrhea associated *C. jejuni* strains at human body temperature (37°C). Whereas bloody/inflammatory diarrhea associated strains grew faster at 42°C (poultry body temperature) compared to 37°C. 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 37°C compared to bloody/inflammatory associated strains. While L-glutamine utilization is significantly higher among bloody/inflammatory diarrhea associated strains at 37°C 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 (37°C) and poultry (42°C) temperatures that may contribute to their differing diarrheal manifestations.

45 **Introduction**

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

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

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

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

94 **Materials and Methods**

95 **Bacterial Culturing and Growth**

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

112 **Growth Curves**

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

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

159 plate reader at either 37°C only (L-fucose and mucin) or both 37°C and 42°C (L-glutamine) with
160 continuous shaking (100 rpm) for 36 hours with OD_{600} reading recorded every 15 minutes. The
161 plate included a blank control well containing 300 µl of uninoculated medium. Results were
162 recorded as the maximum OD_{600} obtained for the culture during 36 hours of growth minus the
163 blank media OD_{600}[39]. Each strain was tested in three biological replicates with three technical
164 replicates for each of the different carbon utilization assays with either L-fucose, mucin or L-
165 glutamine or without supplements (n = 3). There was no growth without any L-fucose, mucin or
166 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 adding
196 1 ml of decolorizer solution (80% ethanol (Fisher Bioreagents) and 20% acetone solution (Fisher
197 Bioreagents)). Finally, 100 µl of the decolorized solution for each strain was transferred to a
198 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

tested for normality (Shapiro–Wilk test) and equal variance (Levene's test). For comparisons between strains associated with watery and bloody/inflammatory diarrhea ($n = 15$ per group), the non-parametric Mann–Whitney U test was applied. Statistical significance was defined as $p \leq 0.05$. For comparisons across multiple strains or conditions, non-parametric Kruskal–Wallis tests with Dunn's post hoc test were used. Compact Letter Display (CLD) notation was used to visualize the results of the pairwise comparisons. Strains that do not share a letter are significantly different from each other ($p < 0.05$), while strains sharing the same letter are not significantly different. Statistical significance was defined as $p \leq 0.05$. Each assay included a minimum of three independent biological replicates per strain ($n = 3$), with technical replicates as noted. Data are presented as mean ± SEM unless otherwise stated.

228 **Results**

229 ***Watery diarrhea-associated C. jejuni strains grow faster than bloody/inflammatory strains at***
230 ***37°C***

231 To evaluate whether differences in growth dynamics could explain divergent clinical
232 manifestations, we compared the growth kinetics of five watery diarrhea-associated and five
233 bloody/inflammatory-associated *C. jejuni* strains at two host-relevant temperatures: 37°C (human)
234 and 42°C (poultry). Growth was monitored over 36 hours using OD_{600} measurements taken every
235 15 minutes. Strain to strain differences were observed widely at both 37°C and 42°C. Strains M129
236 and NCTC11168 grew significantly slower at 37°C than at 42°C ($p < 0.05$; **Figure 1A**).
237 At both temperatures, watery diarrhea associated strains entered logarithmic growth phase earlier
238 and reached higher OD_{600} values compared to bloody/inflammatory strains (**Supplementary**
239 **Figure 1A–D**). One watery strain (D42a) displayed a slower growth profile, similar to that of
240 bloody/inflammatory strains. Generation times calculated from logarithmic growth phase found
241 significantly faster generation times for watery diarrhea associated strains compared to
242 bloody/inflammatory associated strains at 37°C but not at 42°C (37°C watery diarrhea associated
243 115.8 minutes, 37°C bloody/inflammatory diarrhea associated 139.99 minutes $p = 0.018$; 42°C
244 watery diarrhea associated 95.23 minutes, bloody/inflammatory diarrhea associated 42°C 111.47
245 minutes $p = 0.38$; **Figure 1B**). There was no significant difference in generation times at 37°C
246 compared to 42°C for watery diarrhea associated strains ($p = 0.12$) but bloody/inflammatory
247 diarrhea associated strains grew significantly faster at 42°C than 37°C ($p = 0.0057$).

248

249 ***Strains differ in utilization of L-fucose and L-glutamine but not mucin***

250 To determine whether nutrient utilization profiles differ between strains associated with distinct
251 diarrheal outcomes, we screened all ten *C. jejuni* strains for their ability to metabolize 192 carbon
252 sources using BIOLOG PM1 and PM2A phenotype microarray plates. There were a few carbon
253 sources used solely by one or two bloody/inflammatory diarrhea associated strains and not watery
254 associated strains and vice versa, including D-tagatose (D33a), succinnamic acid (D33a, 81-176),
255 citric acid (NCTC11168) and tricatballyic Acid (S3). Several carbon sources exhibited distinct
256 utilization patterns between the two diarrheal manifestation groups (**Supplementary Figure 2**).
257 L-serine was utilized by only three bloody/inflammatory diarrhea associated strains (M129, 81-
258 176 and NCTC11168) and no watery diarrhea associated strains, while L-glycyl-proline was
259 utilized by watery diarrhea associated strains A13a, A14a, S3 but only bloody/inflammatory
260 diarrhea associated strain A9a. Notably, L-glutamine and L-fucose were utilized by multiple
261 strains in only one group: L-glutamine was used by three bloody/inflammatory strains (81-176,
262 M129, D33a) and only one watery strain (S3), while L-fucose was used by four watery strains
263 (A13a, A14a, D42a, S3) and just one bloody/inflammatory strain (A9a). L-fucose and L-glutamine
264 were selected for further phenotypic analysis, along with mucin due to their relevance in host
265 colonization[50-52].
266 All five watery strains showed robust growth in the presence of L-fucose at 37°C (**Figure 2A**).
267 The geometric mean OD_{600} for watery strains was 0.076 compared to 0.030 for
268 bloody/inflammatory strains ($p < 0.0001$). L-fucose supported significantly greater growth in
269 watery diarrhea-associated strains (**Figure 2B**). These results suggest that L-fucose metabolism is
270 a consistent trait among watery diarrhea-associated strains. To assess whether differences in
271 mucosal substrate metabolism extended beyond individual monosaccharides, we next tested the
272 ability of each strain to grow in medium supplemented with bovine submaxillary gland mucin at

273 37°C. Although there was strain-level variability, no significant difference in mucin utilization
274 was observed between watery and bloody/inflammatory strains (p = 0.099), (**Figure 3,**
275 **Supplementary Figure 3**).
276 L-glutamine utilization supported higher levels of growth among all strains compared to L-fucose,
277 with several significant differences between different strains at 37°C or 42°C (**Figure 4A**). Among
278 all strains tested, only A13a showed significantly higher growth at 42°C compared to 37°C, while
279 81-176 and A9a both showed increased growth at 37°C compared to 42°C. However, when
280 grouped by diarrheal manifestation, bloody/inflammatory associated strains exhibited enhanced
281 growth at 37°C in L-glutamine-supplemented medium compared to watery diarrhea associated
282 strains (p = 0.0005) (**Figure 4B**). There was no difference in growth potential at 42°C between
283 watery and bloody/inflammatory strains, (p = 0.68), suggesting a temperature or host dependent
284 role for glutamine utilization among *C. jejuni* strains. Together, these results demonstrate that
285 carbon source utilization differs consistently between strain groups and that these traits may be
286 modulated by host temperature. The enhanced L-fucose metabolism of watery diarrhea associated
287 strains may facilitate rapid growth and colonization in human hosts, while the slower growth and
288 utilization of L-glutamine by bloody/inflammatory diarrhea associated strains contributes to
289 persistence within the human host.
290
291 ***Motility varies widely among C. jejuni strains but does not distinguish diarrheal phenotype***
292 Motility is critically important for *C. jejuni* colonization and pathogenesis, aiding in both adhesion
293 as well as invasion of host cells[53-55]. To assess whether motility differs between strains
294 associated with watery versus bloody/inflammatory diarrhea, we performed swarming motility
295 assays in soft agar at both 37°C and 42°C. A wide range of motility phenotypes was observed

296 among *C. jejuni* strains: bloody/inflammatory strains NCTC11168, D33a, and 81-176, and watery
297 strains D42a and RM1221, were highly motile (>10 mm radius), while bloody/inflammatory strain
298 A9a and watery strain S3 were weakly motile or non-motile (<10 mm) (**Figure 5**). Strains A13a
299 and A14a both had higher (but not significantly different) levels of motility at 42°C. No significant
300 difference in motility was observed between the two diarrheal manifestations at either temperature:
301 37°C ($p = 0.33$) or 42°C ($p = 0.64$; **Supplementary Figure 4**). Additionally, there was no
302 significant difference in motility at different temperatures for the bloody/inflammatory ($p = 0.24$)
303 or watery ($p = 0.35$) diarrhea associated strains.
304
305 ***Biofilm formation does not differ by diarrheal phenotype***
306 Biofilm formation contributes to the persistence, and transmission of *C. jejuni*[56]. Therefore, to
307 assess the potential differences in these traits among our *C. jejuni* strains, we quantified biofilm
308 formation at 37°C using crystal violet staining. Strong biofilm producers ($A_{570} > 0.30$) included
309 bloody/inflammatory diarrhea associated strain M129 and watery diarrhea associated strains A13a
310 and D42a. Medium biofilm formation (A_{570} between 0.20–0.30) was observed in
311 bloody/inflammatory diarrhea associated strains 81-176 and NCTC11168 and watery diarrhea
312 associated strains A14a and S3, while poor biofilm formation ($A_{570} < 0.20$) was noted for A9a,
313 D33a, and RM1221 (**Figure 6A**). Although strains varied in their individual capacities, biofilm
314 formation at 37°C was trending different between diarrheal manifestation groups, but was not
315 significantly different ($p = 0.061$; **Figure 6B**).
316
317
318

319 **Discussion**

320 This study investigated the variation between strains of *C. jejuni* with defined diarrheal phenotypes
321 in the neonatal piglet model, we assessed growth rates, carbon source utilization, motility, and
322 biofilm formation at human and avian body temperatures. In human and poultry hosts, *C. jejuni*
323 must pass through the mucosal layer of the intestines to reach the intestinal epithelial surface and
324 establish an infection. Here, *C. jejuni* strains showed significant strain to strain variation, including
325 differences in the utilization of L-fucose, L-glutamine, generation times, and but did not display
326 significantly different abilities to form biofilms or to utilize bovine submaxillary gland mucin
327 when grouped by diarrheal manifestation, a finding that was not surprising based on *C. jejuni's*
328 limited ability to metabolize carbohydrates[35]. We observed very little to no growth of any of the
329 *C. jejuni* strains in the presence of mucin, suggesting an inability to break it down on their own. It
330 is known that *C. jejuni* growth is enhanced when co-cultured with commensal *Bacteroides*
331 *vulgatus*, a response suggested to be due to the breakdown of mucin by *B. vulgatus*[57]. There are
332 several types of bacteria that are known mucin degraders that have been identified in humans,
333 calves and goats, and include *Bifidobacterium*[58; 59], *Bacteroides*[59] and *Akkermansia*
334 *muciniphila*[60]. Mucin degradation by *A. muciniphila* is known to support intestinal barrier
335 function[61] and provide physiological benefits that prevent disease[61-63]. The composition of
336 mucin degraders in chickens is less well defined, but *Bacteroides* has been found to be one of the
337 most predominating genera of bacteria in the chicken intestinal microbiome[62; 64]. The growth
338 of these commensal bacteria is supported by the highly glycosylated mucins within the intestinal
339 mucus[65], during which active fructosidases are secreted[66; 67]. *C. jejuni* lacks the ability to
340 release L-fucose from glycans and instead is known to utilize free fucose from mucin degraders
341 such as *B. vulgatus*[57].

342 The mucus layer of the intestinal tract is where *C. jejuni* encounters L-fucose, the only known
343 carbohydrate chemoattractant for *C. jejuni*[68; 69]. In the human gut, 80% of the total dry weight
344 of intestinal mucins is made up of *O*-linked glycans[70; 71], however in poultry L-fucose is
345 suggested to comprise an estimated 7% of the *O*-glycans in the intestinal tract[72]. The *fuc* locus
346 for L-fucose catabolism has been identified in >50% of sequenced *C. jejuni* isolates[73] and has
347 been observed among a high percentage of *C. jejuni* isolates from humans[74]. Here we found that
348 strains associated with watery diarrhea exhibited enhanced utilization of L-fucose at 37°C and also
349 grew significantly faster than those linked to bloody/inflammatory disease at 37°C and 42°C. We
350 hypothesis that this combination of rapid growth and efficient metabolism may support early
351 colonization and transmission, contributing to the acute nature of watery diarrhea.
352 The concentration of L-fucose is particularly high among breastfed infants due to the secretion of
353 L-fucose from fucosylated human milk oligosaccharides by intestinal commensals such as
354 *Bifidobacterium* spp and *Bacteriodes* spp[57; 67; 75; 76]. The amount of available L-fucose is
355 known to be as high as 4-5 mg/g in the feces of breastfed infants, and in low to middle income
356 countries, higher proportions of *C. jejuni* have been found among these infants compared to those
357 that were not breastfed[77; 78]. Despite the high levels of available L-fucose, breastfed infants
358 with campylobacteriosis had significantly higher proportions of non-L-fucose metabolizing
359 *Campylobacter* than non-breastfed infants[77], a finding suggested to be driven in part by L-fucose
360 chemotaxis[79].
361 Chemotaxis towards L-fucose in the intestinal lumen through the upregulation of L-fucose
362 dehydrogenase, FucX, is suggested to contribute to its clearance while non-L-fucose metabolizing
363 *C. jejuni* strains move towards the intestinal mucosa to establish disease[79]. These findings
364 support the hypothesis that watery diarrhea associated strains utilize L-fucose in the mucus layer

365 where they are maintained further away from the intestinal epithelial cells while
366 bloody/inflammatory strains move to establish themselves at the enterocyte surface. Indeed, we
367 have recently found that bloody/inflammatory diarrhea associated *C. jejuni* strains are significantly
368 more invasive than watery diarrhea associated strains (Bosquez et al 2025; manuscript currently
369 under review). However, in contrast, L-fucose utilization has also been observed *in vitro* to
370 enhance invasion of Caco-2 cells and significantly increase fibronectin binding efficacy in strain
371 NCTC11168[44]. Another study found similar results with invasion of Caco-2 increased with the
372 addition of 10 mM L-fucose under microaerophilic conditions using *C. jejuni* strain 108[67]. The
373 same study observed growth enhancement of *C. jejuni* strain 108 in the presence of L-fucose, a
374 finding similar to ours, suggesting that watery diarrhea associated strains may alter their
375 invasiveness if tested under the same conditions. In addition, we found that the increased growth
376 potential of watery diarrhea associated strains was maintained at both host-relevant temperatures,
377 suggesting adaptation to colonization across multiple hosts with enhanced growth in humans. The
378 ability to utilize L-fucose provided a colonization advantage *in vivo* in piglets but had no effect on
379 the colonization of chickens when inoculated at a low dose[42]. Since piglets are a model for
380 human disease, whereas chickens are the natural reservoir for *C. jejuni*, this suggests L-fucose
381 utilization at 37°C (human body temperature) versus 42°C (chicken body temperature) may only
382 be relevant to human disease.
383 In contrast, bloody/inflammatory diarrhea-associated strains grew more slowly particularly at
384 37°C, a factor that potentially contributes to prolonged colonization and disease development.
385 Slower growth may allow for slower establishment of an infection, which allows for delayed
386 immune recognition of the pathogen until the bacteria more established, potentially resulting in a
387 more damaging infection. Initial BIOLOG screening found that some strains preferentially utilized

388 L-glutamine, we observed a significant increase in growth potential in the presence of L-glutamine
389 by bloody/inflammatory associated strains compared to watery diarrhea associated strains at 37°C
390 but not at 42°C. In humans, glutamine is known to support epithelial barrier function and modulate
391 host immune responses, and its depletion may promote tissue damage and inflammation[80-82].
392 Because L-glutamine is a key fuel source for enterocytes and supports tight junction integrity[80;
393 83], *C. jejuni* utilization of L-glutamine may not only provide a metabolic advantage but also
394 actively disrupt enterocyte homeostasis. In the context of inflammation, where glutamine demand
395 is heightened due to increased epithelial turnover and immune cell activation, its depletion could
396 contribute to impaired repair to tissue damage as well as reduced immune function[84]. Other
397 studies have found that *C. jejuni* strains have considerable variation in their ability to utilize L-
398 glutamine, with 31% of strains[40] containing the enzyme gamma-glutamyl transpepidase (GGT)
399 necessary for glutamine and glutathione metabolism. One study found a higher percentage of *C.*
400 *jejuni* strains from human isolates (37.93%) to contain the *ggt* gene compared to 14.29% among
401 strains from non-human sources[51], suggesting an advantage for these strains in the human host.
402 Furthermore, a study of 166 *C. jejuni* strains from Finnish patients found an association between
403 bloody diarrhea and GGT production[85], while another study found purified *C. jejuni* GGT
404 impaired intestinal cell and lymphocyte proliferation[86]. The complicated and variable response
405 by *C. jejuni* strains containing *ggt* suggests that individual strains may have complex metabolic
406 requirements or can compensate with alternative pathways not yet characterized. Overall, the
407 metabolic diversity observed among strains highlights the importance of considering host-specific
408 and strain-specific adaptations when investigating L-glutamine utilization among *C. jejuni* strains,
409 especially among those associated with bloody/inflammatory diarrhea.

410 This study found that motility, a critical factor associated with colonization/pathogenicity[87-90],
411 had no significant differences between the different diarrheal manifestation groups. In terms of
412 motility, *Campylobacter* species utilize a single or bipolar flagella to move[91]. Upon entering a
413 host intestinal tract, the ability to move is important for navigating the thick layer of mucus to
414 reach the epithelial surface and colonize[91]. In this study, motility was variable at the strain level
415 and did not correlate strongly with diarrheal manifestations at either 37°C or 42°C in this study.
416 All strains were motile except strain S3, which was originally able to colonize poultry[92; 93], but
417 minimal laboratory passages rendered it defective for colonization[94]. Previously, similar results
418 were demonstrated for non-motile *C. jejuni* strain CS that caused few pathological changes in the
419 piglet model, but grew to a greater maximum cell density in MH medium compared to a
420 genotypically similar but motile strain[88]. Watery diarrhea associated strains with limited to no
421 motility, such as S3, may be able to overcome the mucus layer via the more rapid growth that we
422 observed in this study, but additional studies are needed to confirm this hypothesis. Our results
423 suggest that motility is essential for the pathogenesis of *C. jejuni* regardless of the diarrheal
424 manifestation associated with the strain.
425 Biofilm formation is known to differ among *C. jejuni* strains[95] and has been suggested to
426 promote survival, transmission and colonization of hosts[96; 97]. The present study revealed that
427 there was no significant difference in biofilm formation among watery or bloody/inflammatory
428 diarrhea associated strains at 37°C, suggesting that strains from both groups may have a
429 colonization advantage in humans. In addition to temperature, biofilm formation by bacteria can
430 be triggered by various environmental stressors such as nutrient availability, varying oxygen
431 tensions and osmotic changes[98-100], for example, one study showed that *C. jejuni* biofilm
432 formation was increased under low-oxygen tensions as well as in minimal nutrient medium[101].

433 An enhanced capacity to form biofilms would be particularly useful in surviving the harsh
434 conditions of the intestinal tract such as stomach acid, bile salts, low-oxygen tension, oxidative
435 stress or even immune defenses. When nutrients are available, such as L-fucose, it is known that
436 biofilm formation is reduced, and growth is enhanced *in vitro*[73]. Future studies may investigate
437 the ability of *C. jejuni* strains associated with watery diarrhea to form biofilms when L-fucose is
438 provided in the medium, particularly given their enhanced growth with L-fucose.
439 It is important to note that our study revealed substantial phenotypic variability within each
440 diarrheal manifestation group. This is not unexpected given the known genomic and functional
441 diversity of *C. jejuni*, but it reinforces the need to study multiple strains to identify group-level
442 trends. Within the watery diarrhea group, strain D42a had a longer generation time at 37°C than
443 the other watery associated strains. A13a showed significantly higher growth at 42°C in the
444 presence of L-glutamine. Among bloody/inflammatory strains, NCTC11168, and M129 had
445 slower generation times at 37°C than at 42°C. 81-176, D33a and NCTC-11168 showed similar
446 levels of motility, but A9a and M129 were only weakly. The presence of these outliers highlights
447 the spectrum of metabolic and growth capabilities within each pathotype and suggests that these
448 diarrheal manifestations may require several phenotypes to be present and may also rely on host
449 factors.
450 Another consideration is that strain classification in this study was based on diarrheal outcomes
451 observed in the neonatal piglet model, rather than direct human clinical data. While this may limit
452 immediate generalizability, the piglet model is the only experimental systems that reliably
453 reproduces both watery and bloody/inflammatory diarrheal manifestations caused by *C. jejuni.* It
454 provides a controlled environment to directly associate specific bacterial traits with clinical
455 outcomes, eliminating many of the confounding variables inherent in human observational studies.

456 Additionally, clinical strains are likely biased towards bloody/inflammatory diarrhea associated
457 strains, as people suffering from the less severe watery diarrhea are not likely to seek medical
458 attention. Nonetheless, validation of these findings using well-characterized human isolates with
459 known clinical presentations will be important for confirming their broader relevance.
460 Despite these limitations, our findings provide evidence that clinical outcome in *C. jejuni* infection
461 may be shaped by fundamental differences in bacterial growth and metabolism. Strains associated
462 with bloody/inflammatory diarrhea are slower growers, utilize L-glutamine for enhanced growth
463 at 37°C, while those linked to watery diarrhea appear optimized for rapid expansion and mucus-
464 derived sugar utilization. These strategies may reflect differing host adaptation trajectories and
465 suggest that metabolic profiling could provide new insight into the pathogenic potential of *C. jejuni*
466 lineages.
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Contributors

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

Conflicts of Interest

The authors have no conflicts of interest.

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494 **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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508 **Figure Legends**

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

522

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

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

553 **Figure 5. Motility at 37°C and 42°C.** Average motility of *C. jejuni* strains after 48-hour
554 incubation at both 37°C and 42°C. Motility was measured as the radius of the halo of growth in
555 millimeters after 48-hour incubation. Average motility was calculated from three biologically
556 independent assays with two technical replicates (n = 3). Compact letter displays (CLDs) indicate
557 statistically significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis
558 test followed by Dunn's post hoc test (n = 3). Bars labeled with different letters indicate statistically
559 significant difference ($p < 0.05$) between those strains and/or temperatures, whereas shared letters
560 denote groups that are not significantly different from each other.
561
562 **Figure 6. Biofilm production at 37°C.** (A) Formation of biofilm after 72 hours at 37°C among
563 *C. jejuni* strains. Biofilm formation was quantified using spectrometer readings at 570 nm
564 following a 72-hour incubation at 37°C. Compact letter displays (CLDs) indicate statistically
565 significant differences between groups ($p < 0.05$) as determined by Kruskal-Wallis test followed
566 by Dunn's post hoc test. Bars labeled with different letters indicate statistically significant
567 difference ($p < 0.05$) between those strains, whereas shared letters denote groups that are not
568 significantly different from each other. Average biofilm formation was determined from three
569 biologically independent assays at 37°C with two technical replicates (n = 3) (B) Average biofilm
570 production of bloody/inflammatory and watery diarrhea associated strains after 72 hours at 37°C.
571 Average biofilm production at 37°C was calculated from three biologically independent assays
572 with two technical replicates (n = 15).
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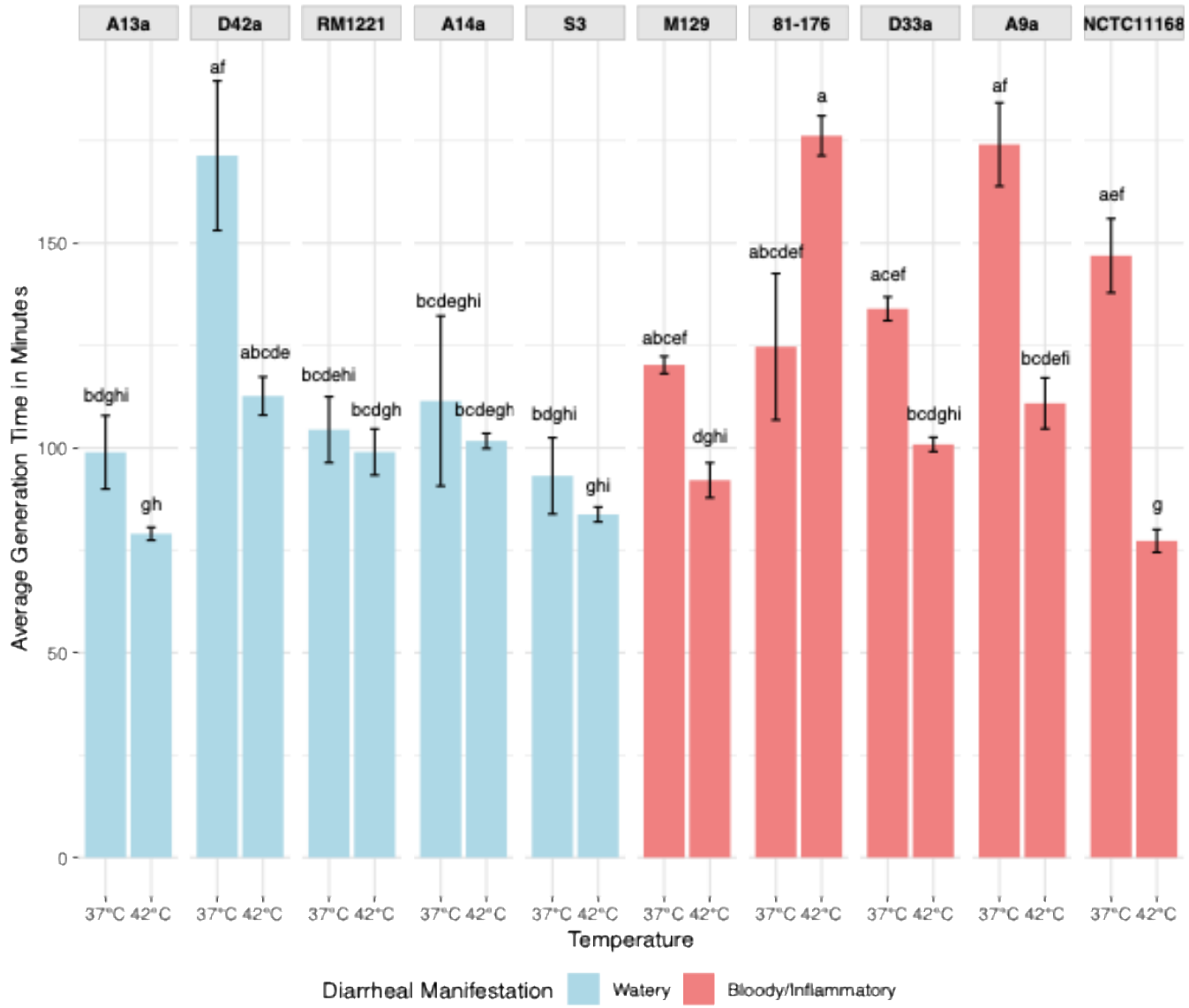
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Watery
Bloody/Inflammatory

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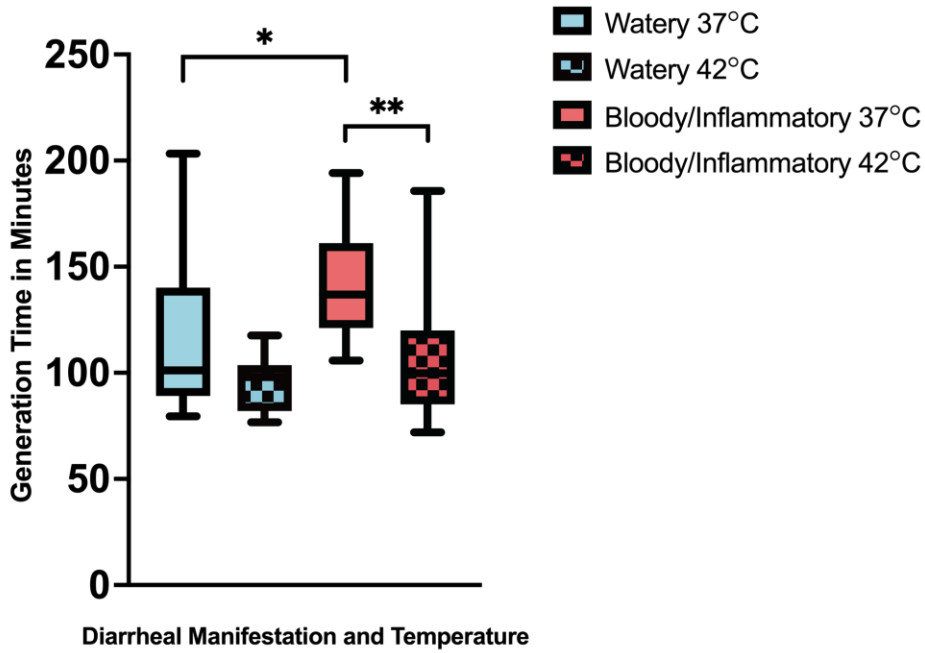
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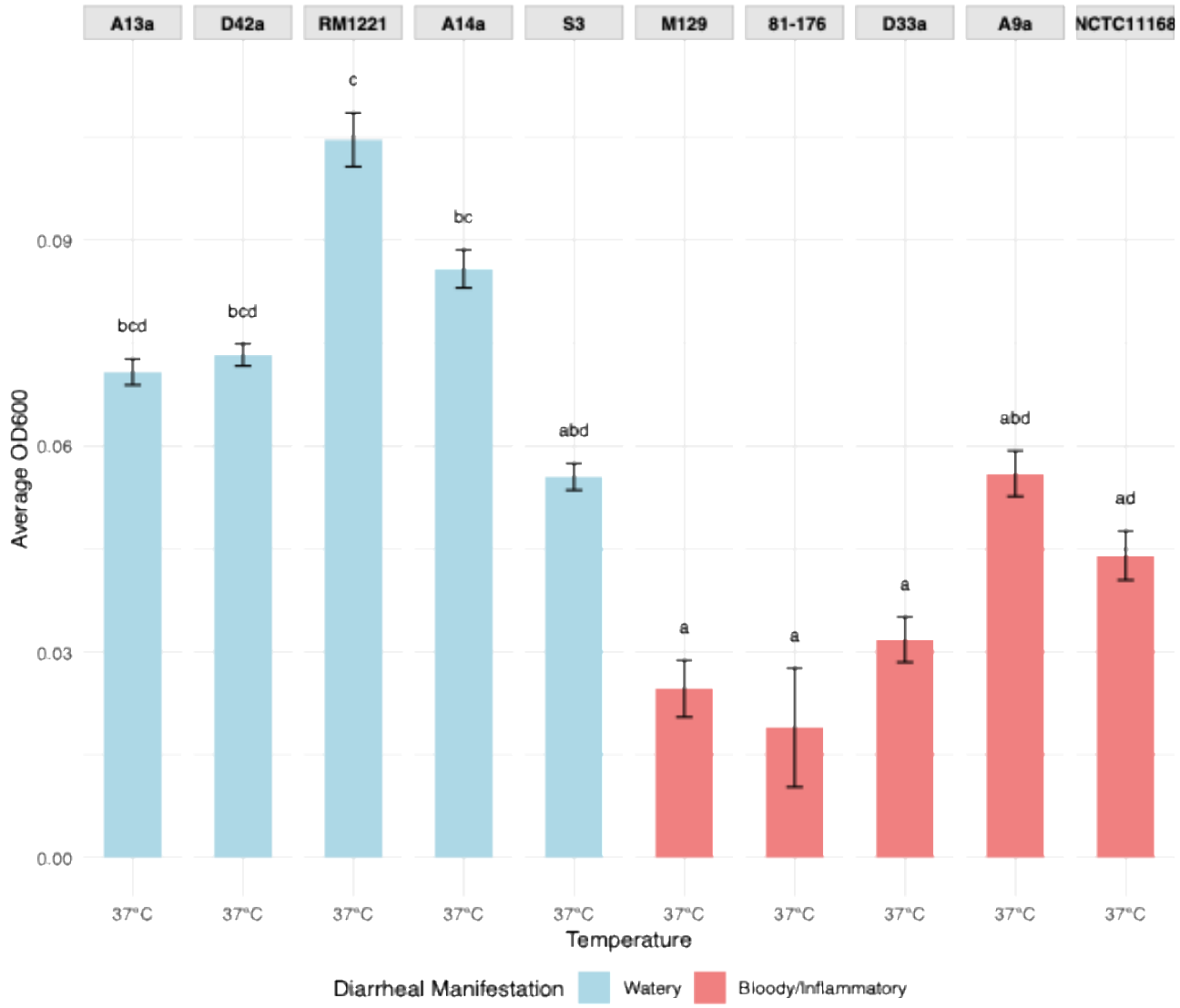
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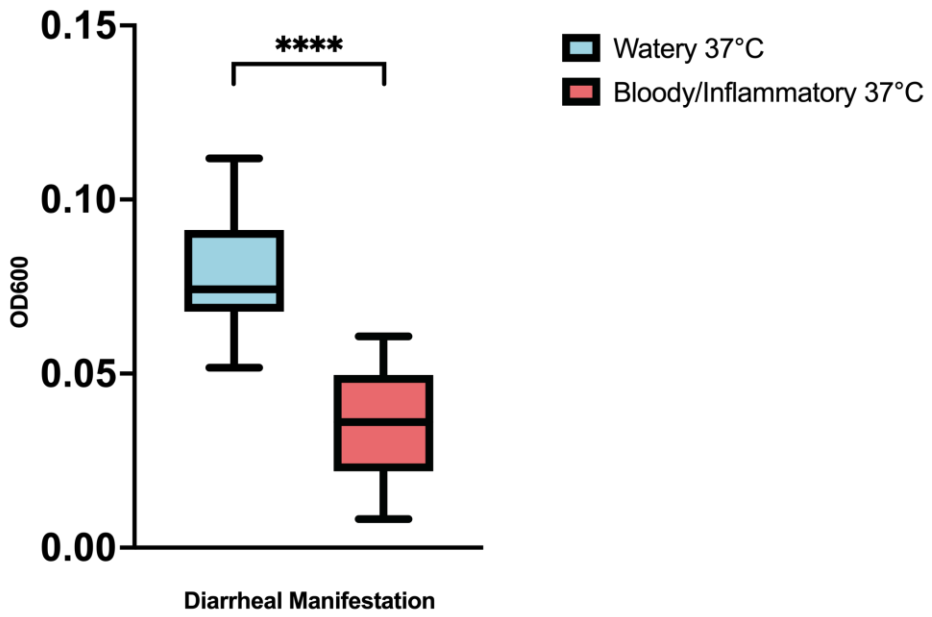
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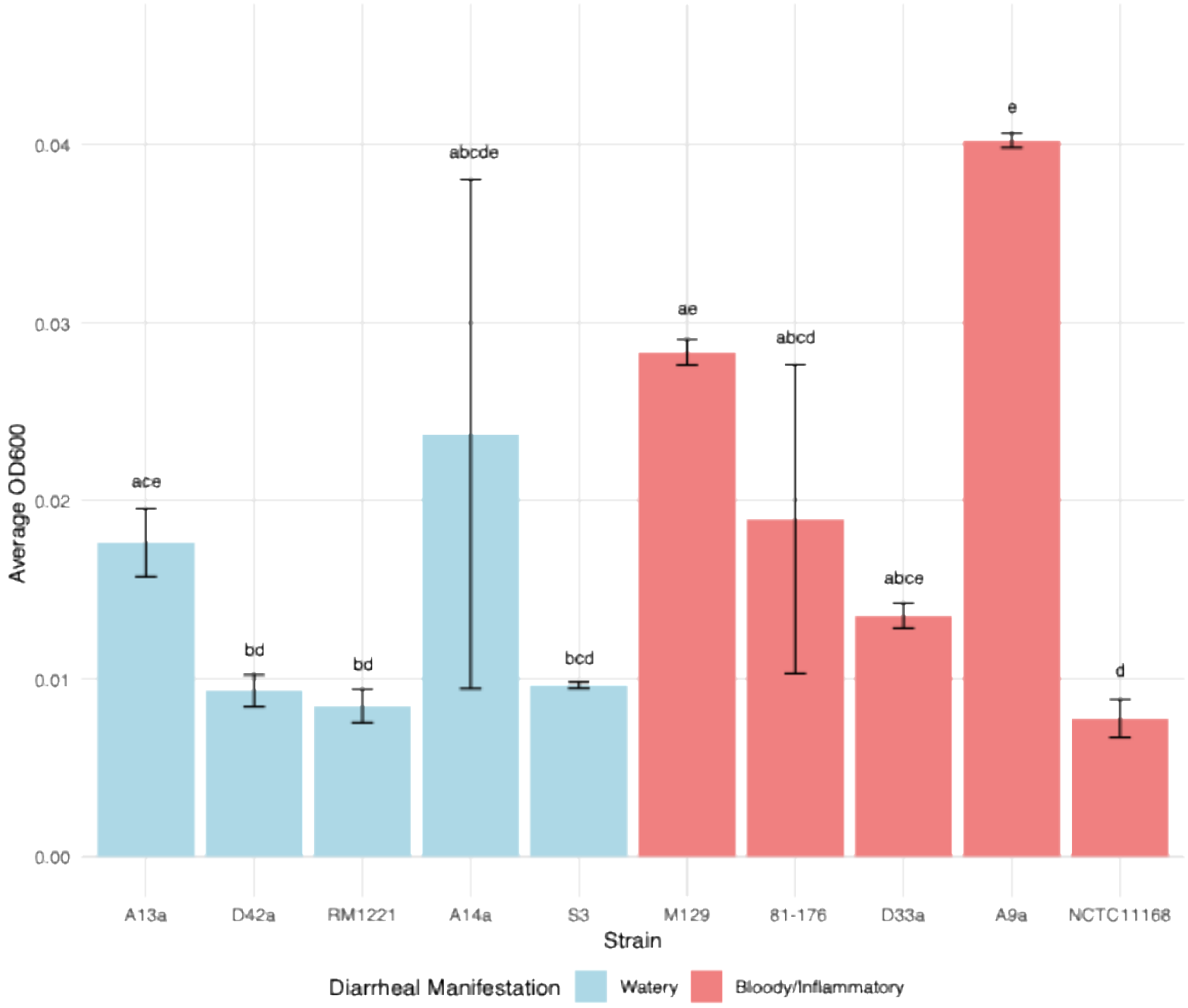
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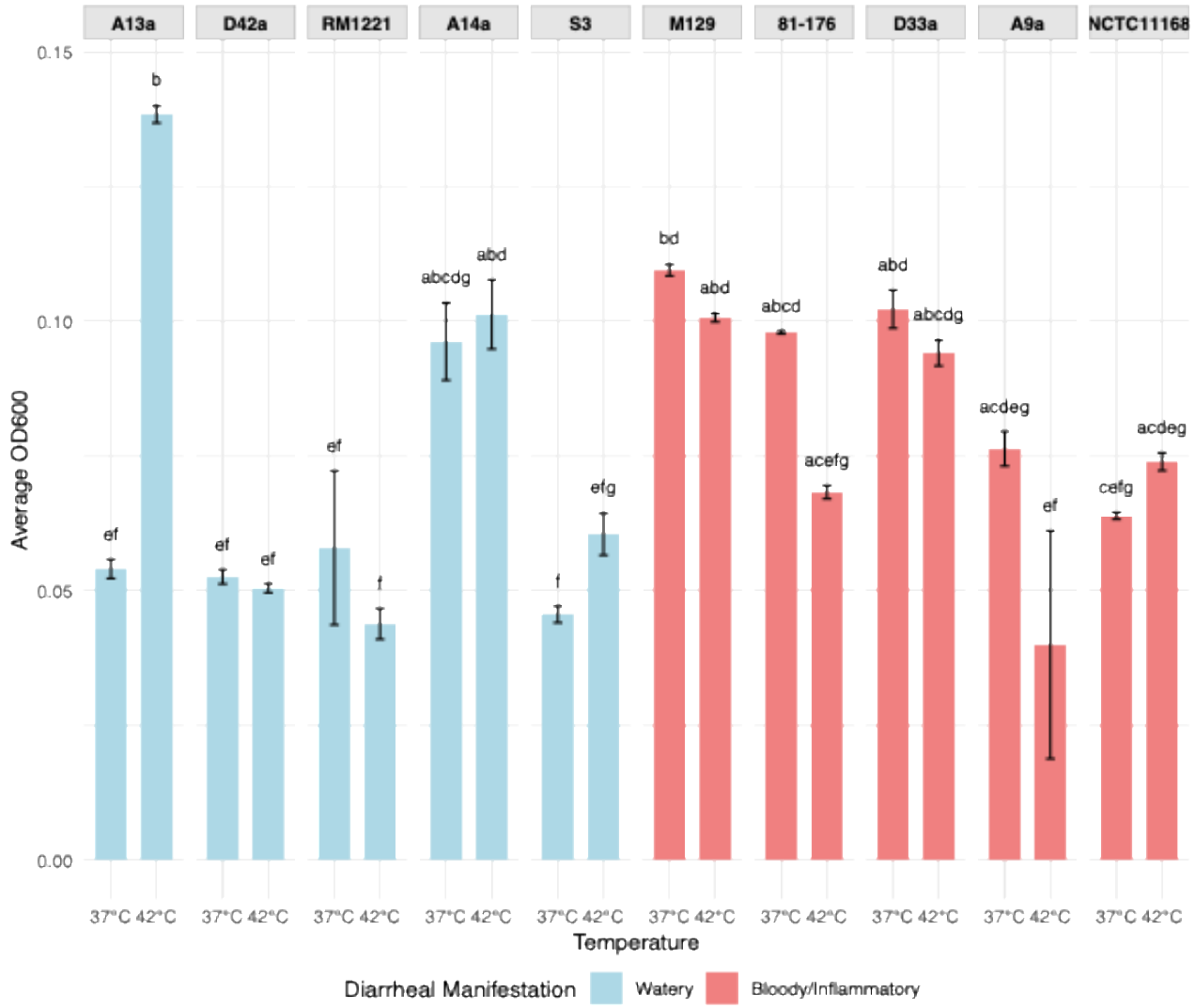
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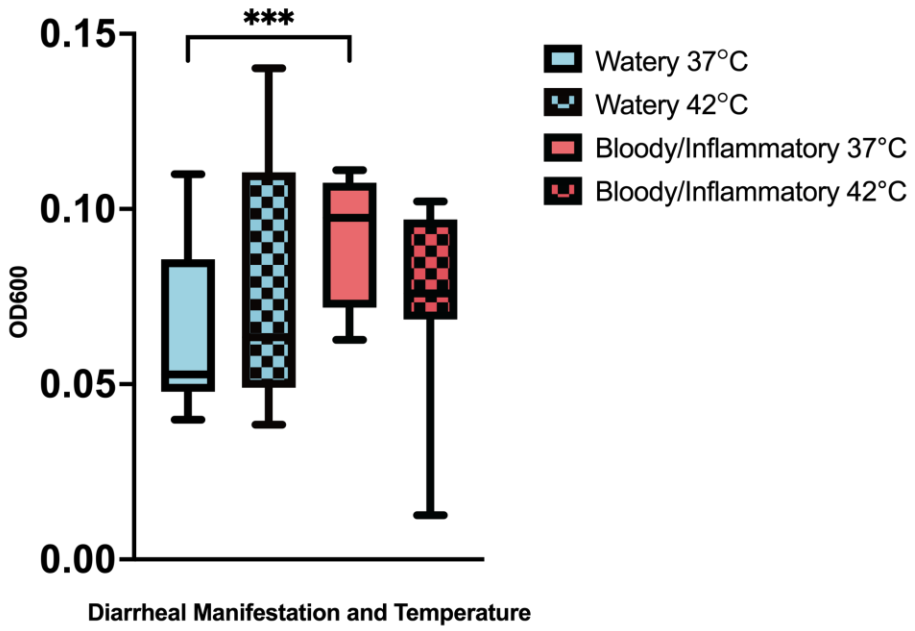
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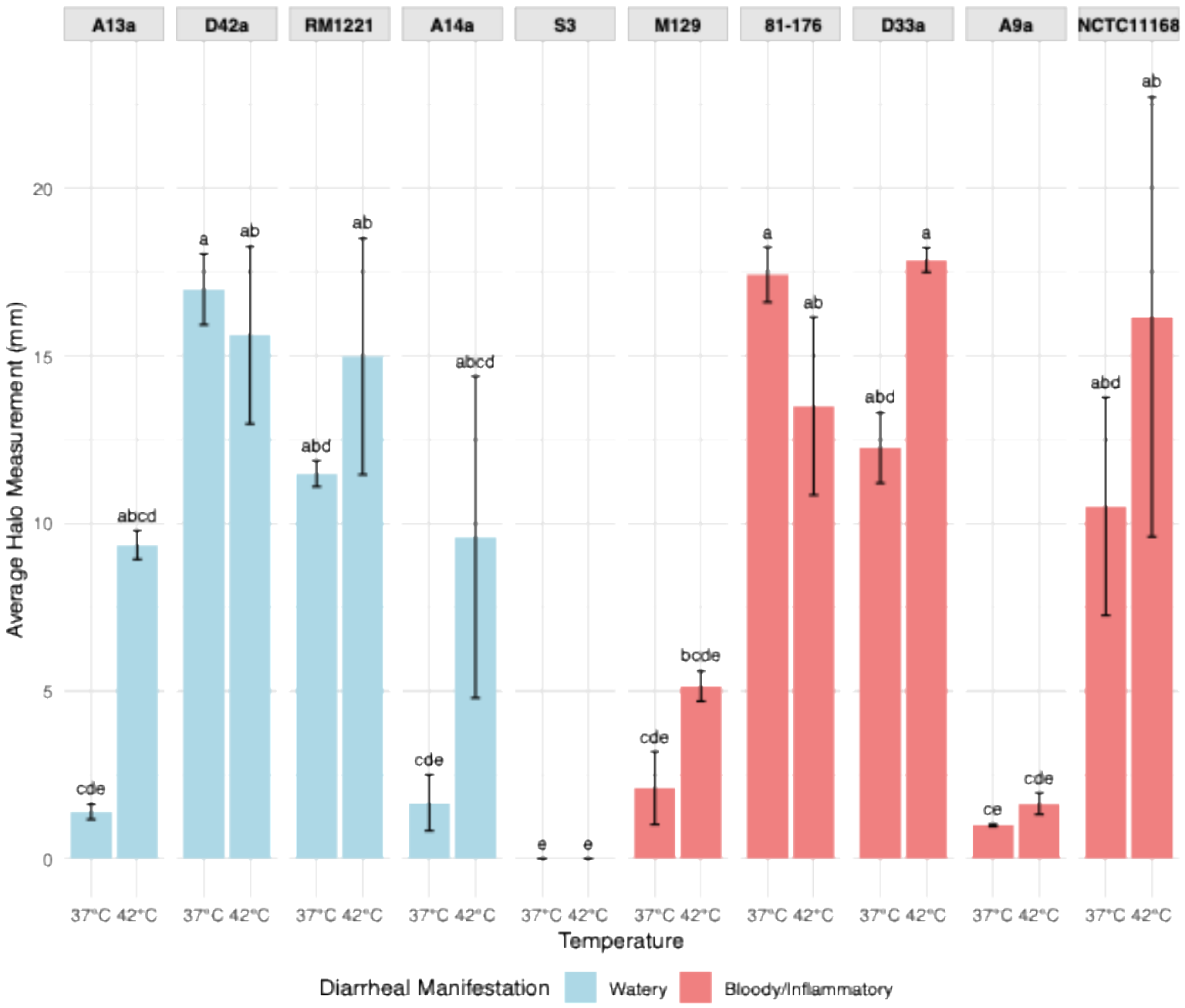
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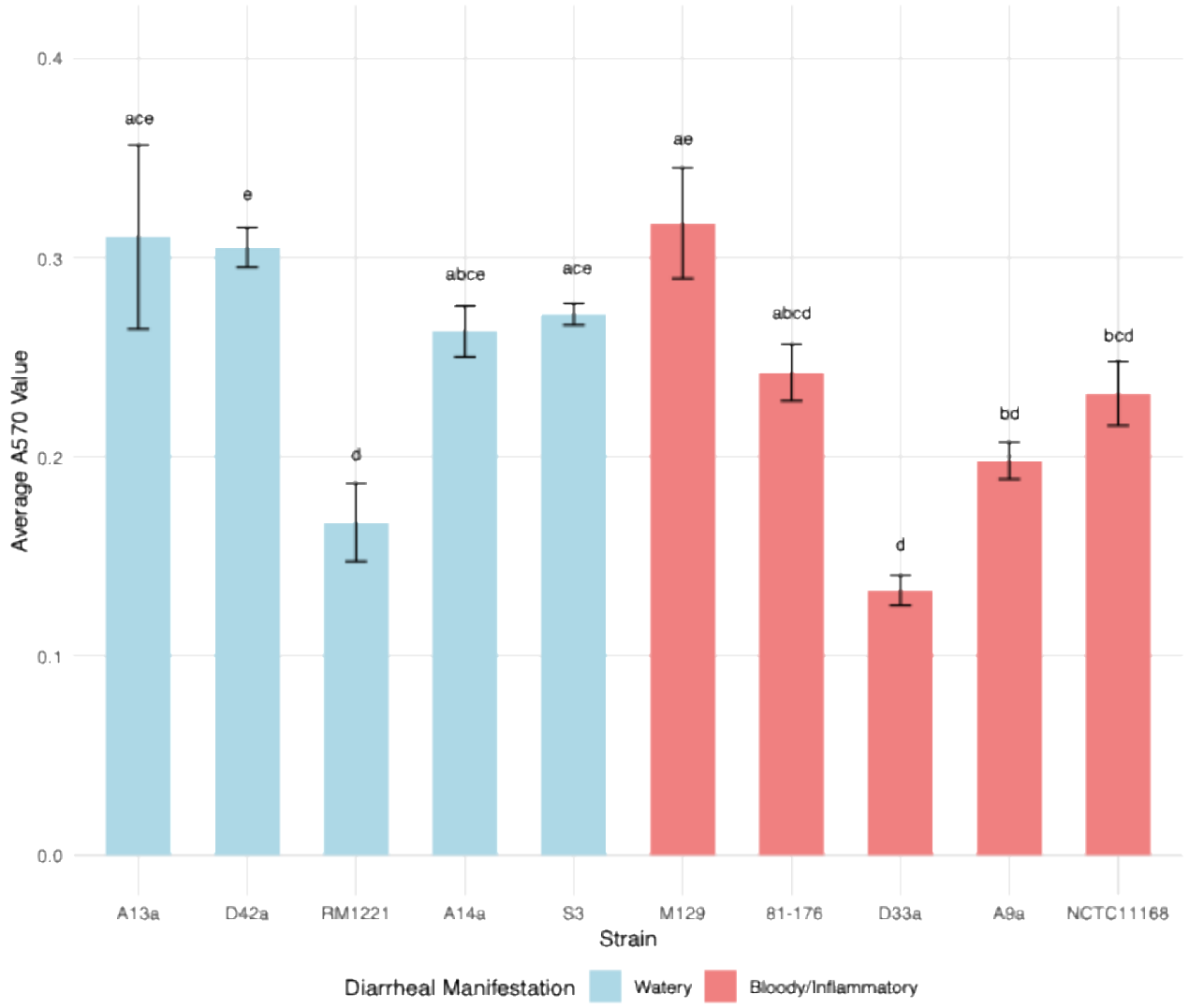
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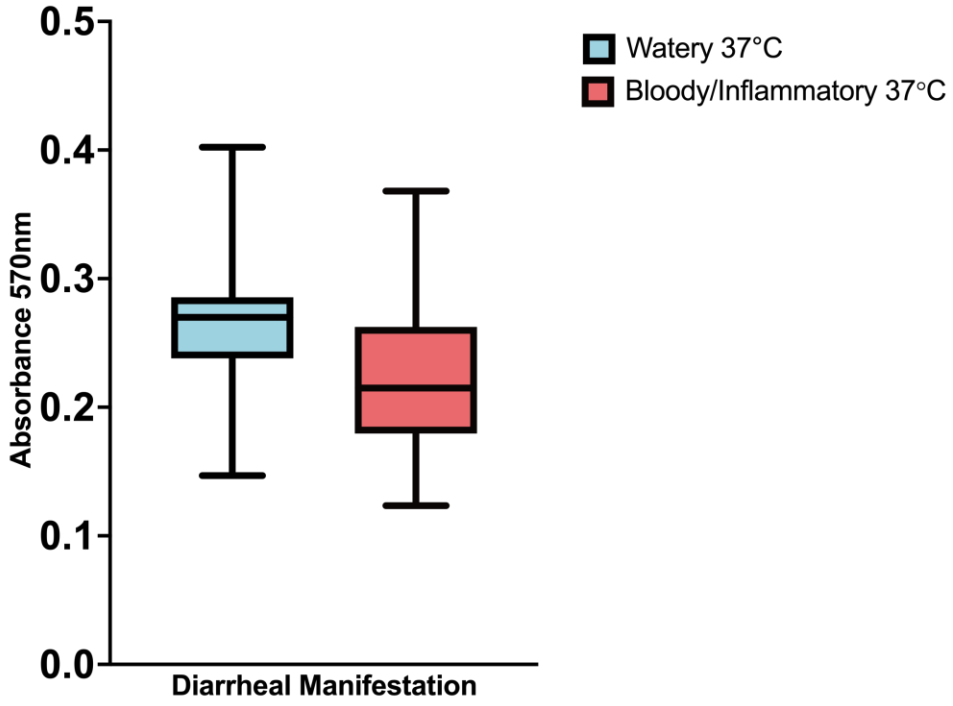
Bloody/Inflammatory

B


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance 570nm
Diarrheal Manifestation
Watery 37°C
Bloody/Inflammatory 37°C