Strain-specific virulence signatures of *Campylobacter jejuni* associated with watery versus bloody diarrhea in the neonatal piglet model

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Abstract

Campylobacter jejuni is the leading cause of bacterial gastroenteritis globally, presenting with either watery or bloody/inflammatory diarrhea. Despite this clinical variability, the underlying mechanisms driving these distinct diarrheal manifestations remain unclear. The neonatal piglet model provides a unique opportunity to investigate these differences using strains with defined disease manifestations. We evaluated ten *C. jejuni* strains previously shown to cause consistent diarrheal manifestations in neonatal piglets: five strains associated with watery diarrhea and five associated with bloody/inflammatory diarrhea. The study found significant strain to strain variation among most assays, but overall strains associated with bloody/inflammatory diarrhea exhibited significantly higher levels of epithelial cell invasion, intracellular survival, macrophage survivability, and disruption of intestinal barrier integrity compared to watery diarrhea strains. Notably, both groups had similar levels of adherence, produced similar levels of cytolethal distending toxin (CDT) and elicited comparable IL-8 responses. While both groups facilitated translocation of commensal *E. coli*, only bloody/inflammatory strains caused increased paracellular permeability, as shown by TEER and FITC-dextran assays. This study reveals that there is significant variation in the virulence phenotype of *C. jejuni* strains and identifies several characteristics consistently associated with a specific diarrheal manifestation group. These findings provide new insights into the strain-specific pathogenic mechanisms of *C. jejuni*, with implications for understanding host-pathogen interactions and informing targeted diagnostic and therapeutic strategies.

Author Summary

Campylobacter jejuni is one of the most common bacterial causes of diarrhea worldwide, affecting millions each year. Infected individuals may experience either watery or bloody/inflammatory diarrhea, but the reasons why different strains cause different types of illness have remained a mystery. In this study, we used a neonatal piglet model, which accurately mimics human diarrheal disease, to investigate why certain *C. jejuni* strains cause more severe symptoms than others. We compared strains known to cause either watery or bloody diarrhea and found key differences in their ability to invade intestinal cells, survive inside immune cells, and damage the intestinal barrier. While both groups of strains could trigger inflammation and translocate *E. coli* through the intestinal lining, only the strains linked to bloody diarrhea caused significant barrier disruption and had increased intracellular survival. These findings reveal that different *C. jejuni* strains follow distinct paths during infection, which helps explain why they produce different symptoms. Unraveling these differences improves our overall understanding of *Campylobacter* pathogenesis that could lead to improved diagnostics and/or treatments to reduce the burden of campylobacteriosis around the world.

66 **Introduction**

67 *Campylobacter jejuni* is the leading cause of bacterial gastroenteritis worldwide,
68 responsible for over 550 million cases annually[1]. In the United States alone, it affects more than
69 1.9 million people per year, imposing significant economic and public health burdens[2]. While
70 typically self-limiting, *C. jejuni* infection can lead to long-term sequelae such as post-infectious
71 irritable bowel syndrome (PI-IBS), reactive arthritis, and Guillain-Barré syndrome[3-5].
72 Increasing evidence also links *Campylobacter* to growth stunting in children from low- and
73 middle-income countries due to chronic enteric infections[6-8].

74 Clinically, *C. jejuni* infection presents as watery or bloody/inflammatory diarrhea, with up
75 to 27% of U.S. cases involving visible blood in stools[9]. Bloody/inflammatory diarrhea suggests
76 deeper intestinal invasion and inflammation, often associated with more severe outcomes[10].
77 Despite decades of reporting this clinical distinction, the mechanisms driving these divergent
78 diarrheal manifestations remain poorly understood. Several virulence factors have been proposed
79 to influence disease severity, including enterotoxin production, cytotoxins like cytolethal
80 distending toxin (CDT), and bacterial invasiveness[11-13]. However, no studies have definitively
81 linked these traits to specific diarrheal outcomes. Clinical data are often confounded by subjective
82 patient reporting and variable strain backgrounds, complicating direct comparisons. Additionally,
83 many *Campylobacter* studies rely heavily on just a few clinical *C. jejuni* strains, most commonly
84 NCTC11168, 81-176, or 81116[14, 15], which may unintentionally bias findings, given the well-
85 documented heterogeneity in virulence factors across *C. jejuni* strains virulence factors[16-18].

86 The neonatal piglet model provides a robust platform for studying *C. jejuni*-induced
87 diarrhea[19]. Unlike murine models, piglets exhibit human-like disease and can consistently
88 replicate both watery and bloody/inflammatory diarrheal manifestations following infection with

specific *C. jejuni* strains[20, 21]. Additionally, piglets are recognized as strong alternatives to both canines or non-human primates for non-rodent evaluations that are required by regulatory agencies[22, 23], and also have intestinal functions physiologically similar to humans[24-26]. Utilization of this model allows for controlled, strain-specific comparisons with minimized host variation. Healthy piglets typically do not develop disease when exposed to *C. jejuni*[27]. However, colostrum-deprived neonatal piglets consistently develop clinical symptoms such as large intestinal lesions, inflammation, and diarrhea lasting up to six days depending on the *C. jejuni* strain used for infection[28]. Similarly, gnotobiotic piglets infected with *C. jejuni* also display symptoms of diarrhea and sloughing of epithelial cells from the mucosa in the cecum and colon closely resembling human campylobacteriosis pathologies[20, 29]. Despite these advantages, the use of piglets in *C. jejuni* research remains limited due to the high costs and specialized housing requirements, leading many researchers to favor more accessible models such as mice, ferrets, guinea pigs, or rats.

In this study, we leveraged the neonatal piglet model to investigate phenotypic differences between *C. jejuni* strains that cause watery diarrhea compared to those that cause bloody/inflammatory diarrhea. Using ten strains classified by the resultant diarrheal manifestation, we assessed virulence traits including invasion, intracellular survival, epithelial barrier disruption, and immunostimulation. Our goal was to evaluate these virulence and pathogenicity traits that may relate to the differences in diarrheal presentation, advancing our understanding of *C. jejuni* pathogenesis and informing new approaches to diagnosis or intervention.

112 **Materials and Methods**

113 **Bacterial Culturing and Growth**

114 *C. jejuni* strains used in this study are listed in **Table 1**. All strains were grown and maintained on
115 Mueller-Hinton agar plates supplemented with 5% defibrinated bovine blood (MHB; Becton,
116 Dickinson and Company Difco) for 48 hours at 37°C with 10% CO_2. Strains for assays that
117 required liquid culture were grown in Mueller-Hinton broth (MH; Becton, Dickinson and
118 Company Difco) with constant shaking (100 rpm) for 16-18 hours at 37°C with 10% CO_2. Assay
119 control strains *Escherichia coli* DH5α and *Salmonella enterica* subspecies *enterica* serovar
120 Typhimurium str. RM6835 were both grown aerobically either on Luria-Bertani (LB) agar (Fisher
121 Bioreagents) at 37°C for 24 hours or in LB broth (Fisher Bioreagents) with constant shaking (100
122 rpm) at 37°C for 24 hours. For selective isolation of *Campylobacter* in select assays, *C. jejuni*
123 strains were cultured on *Campylobacter* CEFEX agar, a modified MH agar with the addition of
124 vancomycin (20 mg/L), polymyxin B (0.35 mg/L), trimethoprim (26.4 mg/L), sodium
125 cefoperazone (20 mg/L) and amphotericin B (10 mg/L).

126

127 **Cytolethal Distending Toxin (CDT) Assay**

128 CDT activity was measured using a modified protocol from Abuoun et al[30]. Briefly, *C. jejuni*
129 strains were grown overnight on MHB plates at either 37°C or 42°C under microaerobic conditions
130 (10% CO_2). Bacterial growth was harvested by resuspending the entire plate in 5 ml of *Brucella*
131 broth (Becton, Dickinson and Company, BBL). The suspension was centrifuged at 10,000 × g for
132 20 minutes at 4°C. The supernatant was discarded, and the bacterial pellet was resuspended in 3
133 ml of Eagle's Minimum Essential Medium (EMEM; Corning) supplemented with 10% fetal
134 bovine serum (FBS; Corning). The OD_{600} of the suspension was adjusted to 1.0 using EMEM +

135 10% FBS. Lysates were prepared by sonicating the suspension on ice using a QSonica sonicator
136 (20-second pulses, 20-second rests, five cycles total). The lysate was centrifuged at 10,000 × g for
137 20 minutes at 4°C, and the supernatant was filter-sterilized using a 0.22 µm syringe filter (Fisher
138 Scientific) to remove viable bacteria. Sterile lysates were serially diluted two-fold (1:1 to 1:128)
139 in EMEM + 10% FBS. HeLa cells (ATCC CCL-2) were seeded into 96-well plates at 2×10^4
140 cells/ml in 100 µl of EMEM + 10% FBS and incubated for 3 hours at 37°C with 5% CO_2.
141 Subsequently, 100 µl of each dilution of filtered cell lysate was added to the HeLa cells. Plates
142 were incubated at 37°C with 5% CO_2 for five days. Following incubation, cells were fixed with
143 10% formalin and stained with 0.8% crystal violet. The CDT titer was defined as the reciprocal of
144 the highest dilution showing >50% cell distension. *E. coli* DH5α was used as a negative control.
145 Each strain was tested in three biologically independent assays (n = 3).

146

147 **Attachment Assays**

148 Attachment was assessed using three epithelial cell lines: non-polarized INT407 (ATCC CCL-6),
149 and polarized Caco-2 BBE (ATCC HTB-37) and T84 (ATCC CCL-248) cells. Cell lines were
150 seeded in 24-well tissue culture-treated plates (1 ml per well at 1×10^6 cells/ml) and incubated for
151 18 hours at 37°C with 5% CO_2. *Campylobacter* cultures were grown overnight in MH broth with
152 shaking (100 rpm) at 37°C in 10% CO_2. The OD_{600} was adjusted to 0.1 in the appropriate medium
153 for each cell line: EMEM + 10% FBS for INT407, EMEM + 20% FBS for Caco-2 BBE, and
154 DMEM/F-12 + 5% FBS for T84. Each well was washed twice with sterile PBS, then inoculated
155 with 1 ml of *C. jejuni* inoculum (approximately 1×10^8 CFU, multiplicity of infection (MOI) 100).
156 To enhance bacterial contact with host cells, plates were centrifuged at 600 × g for 5 minutes then
157 incubated for 1 hour at 37°C with 5% CO_2. Wells were then washed twice with PBS to remove

158 non-adherent bacteria. Host cells were lysed by adding 1 ml of 0.1% Triton X-100 in PBS and
159 incubating for 10 minutes at room temperature. Lysates were serially diluted and plated on MHB
160 agar for CFU enumeration after incubation at 37°C in 10% CO_2 for 48 hours. Attachment
161 percentage was calculated as: (CFU recovered / CFU inoculated) × 100. Each strain was tested in
162 technical duplicates in three independent experiments (n = 3). Negative controls included two
163 wells inoculated with only sterile PBS. *E. coli* DH5α was used as a positive control. Due to
164 insufficient T84 cell growth, *C. jejuni* strain 81-176 was unable to be included in attachment assays
165 using this line.
166
167 **Invasion assays**
168 Enterocyte invasion was assessed using INT407, Caco-2 BBE, and T84 cells, following the same
169 seeding and infection conditions described for attachment assays. After overnight growth of *C.*
170 *jejuni* in MH broth at 37°C with 10% CO_2 and shaking (100 rpm), cultures were diluted to OD_{600}
171 = 0.1 in the appropriate medium for each cell line to achieve a MOI of 100. Each well of the 24-
172 well plates was washed twice with PBS and then inoculated with 1 ml of the bacterial suspension.
173 Plates were centrifuged at 600 × g for 5 minutes and incubated for 3 hours at 37°C with 5% CO_2
174 to allow for bacterial invasion. Following incubation, wells were washed twice with PBS, and 1
175 ml of cell line-specific medium containing gentamicin (250 µg/ml; Acros Organics) was added to
176 each well. Plates were incubated for an additional hour to eliminate extracellular bacteria. After
177 gentamicin treatment, wells were washed twice with PBS and then lysed with 1 ml of 0.1% Triton
178 X-100 in PBS. The lysates were serially diluted and plated on MHB agar, which was incubated at
179 37°C with 10% CO_2 for 48 hours. The percent invasion was calculated as: (CFU recovered after
180 gentamicin / CFU in initial inoculum) × 100. Each strain was assessed in technical duplicates in

181 three biologically independent experiments (n = 3). *Salmonella* Typhimurium strain RM6835 was
182 used as a positive control, and *E. coli* DH5α as a negative control. Every assay included two
183 uninoculated wells and were processed exactly as the positive and negative controls. Due to
184 insufficient T84 cell growth, *C. jejuni* strain 81-176 was unable to be included in invasion assays
185 using this cell line.
186
187 **Macrophage Survivability**
188 The ability of the different *C. jejuni* strains to survive intracellularly in macrophages was assessed
189 using J774A.1 mouse macrophages (ATCC TIB-67). Cells were seeded into 24-well plates at $1 \times$
190 10^6 cells/ml in 1 ml per well and incubated for 18 hours at 37°C with 5% CO_2 to establish
191 monolayers. Overnight MH broth cultures of each *C. jejuni* strain were prepared, adjusted to OD_{600}
192 = 0.1 (~1×10^8 CFU/ml) in DMEM + 10% FBS (J7774A.1 culture media), and added to each well
193 at an MOI of 100. Two wells were left uninoculated as negative controls. Plates were incubated at
194 37°C with 5% CO_2 for 24, 48, or 72 hours. At each time point, wells were washed twice with PBS
195 and then treated with 1 ml of gentamicin (250 µg/ml in DMEM + 10% FBS) for 1 hour to eliminate
196 extracellular bacteria. After treatment, wells were washed again, and macrophages were lysed by
197 adding 1 ml of 0.1% Triton X-100 in PBS. Lysates were serially diluted and plated on MHB agar,
198 incubated at 37°C with 10% CO_2 for 48 hours, and colonies enumerated. Each strain was tested in
199 two technical replicates across three independent biological assays (n=3). *C. jejuni* strain 81-176
200 was included as a positive control due to its known interaction with macrophages[31].
201
202 **IL-8 ELISA and 24-hour Intracellular Survivability**

203 T84 cells were seeded into 24-well plates at 5.5×10^5 cells/well and cultured for approximately 7
204 days until reaching 100% confluence. *C. jejuni* strains were grown overnight in MH broth and
205 diluted to $OD_{600} = 0.1$ in DMEM/F-12 with 5% FBS (T84 medium). Each well was infected with
206 1 ml of bacterial suspension (~1×10^8 CFU, MOI 100) and incubated at 37°C with 5% CO_2 for 3
207 hours. Following infection, wells were washed with PBS and treated with 1 ml of gentamicin (250
208 µg/ml in T84 medium) for 1 hour. Wells were then washed again, fresh medium was added, and
209 plates were incubated for 24 hours at 37°C with 5% CO_2. After 24 hours, media from each well
210 was collected, centrifuged at 12,000 × g for 1 minute, and the supernatant stored at –80°C for
211 cytokine analysis. Human IL-8 levels were measured using the BD OptEIA™ ELISA kit (BD
212 Biosciences) per the manufacturer's instructions. To assess intracellular bacterial survival,
213 epithelial cells were lysed after supernatant removal by adding 1 ml of 0.1% Triton X-100 in PBS.
214 Lysates were serially diluted and plated on MHB agar. Plates were incubated at 37°C in 10% CO_2
215 for 48 hours, and colonies were counted. Each strain was evaluated in two technical replicates per
216 assay, with three independent biological replicates (n = 3). Due to insufficient T84 cell growth, *C.*
217 *jejuni* strain 81-176 was unable to be included in IL-8 and intracellular assays using this cell line.
218

219 **Transepithelial Electrical Resistance (TEER)**

220 To evaluate epithelial barrier integrity, transepithelial electrical resistance (TEER) was measured
221 using Caco-2 BBE cells. Cells were seeded at 2×10^5 cells per well into 0.4 µm pore-size Transwell
222 inserts (Falcon, Corning) placed in 24-well tissue culture-treated plates. Cells were cultured in
223 EMEM with 20% FBS at 37°C with 5% CO_2 until a confluent monolayer formed (TEER > 250
224 $\Omega \cdot cm^2$), typically by day 14. Monolayer integrity was assessed microscopically and with an
225 EVOM2 volt/ohm meter (World Precision Instruments). Following confluence, cells were

226 maintained for an additional 120 hours to promote tight junction maturation. Prior to infection,
227 TEER was recorded, and a cell count was performed using a hemocytometer to calculate the
228 bacterial concentration needed for MOI 100. *C. jejuni* cultures were prepared by growing strains
229 overnight in MH broth, adjusting to OD_{600} = 0.1, and diluting in Caco-2 BBE medium. From each
230 Transwell, 50 µl of media was removed from the apical compartment and replaced with 50 µl of
231 the corresponding *C. jejuni* inoculum (~5 × 10^6 CFU; MOI 100). Each strain was tested in duplicate
232 Transwells, with two uninoculated wells serving as negative controls (sterile medium only).
233 Infected plates were incubated at 37°C with 5% CO_2 for 72 hours. TEER measurements were taken
234 at 24, 48, and 72 hours using Millicell ERS-2 electrodes (Millipore). Electrodes were disinfected
235 and rinsed between each well using the following sequence: 10% bleach, sterile water (×2), 70%
236 ethanol, sterile water (×2), and pre-warmed media. Each *C. jejuni* strain was tested in duplicates
237 during three independent biological experiments (n = 3).
238
239 **FITC-Dextran Translocation**
240 To assess paracellular permeability to small molecules, a 4-kDa fluorescein isothiocyanate (FITC)-
241 dextran probe (Millipore Sigma) was applied to mature Caco-2 BBE monolayers grown on 0.4 µm
242 Transwell inserts in phenol red-free MEM (Gibco). Cells were seeded at 2 × 10^5 cells per insert
243 and cultured to confluence (TEER > 250 Ω·cm^2), then maintained for an additional 120 hours to
244 allow tight junction maturation. Overnight *C. jejuni* cultures were adjusted to OD_{600} = 0.1 (~5 ×
245 10^6 CFU) and used to inoculate the apical compartments (50 µl per insert, MOI 100). Two wells
246 were left as negative controls (sterile medium only). After 72 hours of incubation at 37°C with 5%
247 CO_2, 100 µl of media was removed from the apical compartment and replaced with 100 µl of 4-
248 kDa FITC-dextran solution (10 mg/ml in PBS). Plates were incubated for 3 hours, then 100 µl was

249 collected from the basolateral compartment and transferred to a black walled 96-well plate for
250 fluorescence measurement. Fluorescence was measured using a SpectraMax M2 plate reader
251 (Molecular Devices) with excitation at 490 nm and emission at 530 nm. Relative fluorescence
252 units (RFUs) were blanked against sterile Caco-2 BBE medium. Each *C. jejuni* strain was tested
253 in three biological experiments with two technical replicates each (n = 3).
254
255 **Commensal Bacteria Translocation**
256 The ability of *C. jejuni* to disrupt the epithelial barrier and promote translocation of non-invasive
257 commensal bacteria was assessed using *E. coli* DH5α as a model commensal. Two experimental
258 approaches were performed: (1) a 6-hour co-inoculation and (2) a 72-hour pre-infection with *C.*
259 *jejuni* followed by 3-hour *E. coli* exposure. For both approaches, Caco-2 BBE cells were seeded
260 at 1.5×10^5 cells/ml into 3 µm pore-size Transwell inserts and cultured to confluence (TEER >
261 250 $\Omega \cdot cm^2$), with daily monitoring of TEER.
262 ***6-hour co-culture:*** Overnight cultures of *C. jejuni* and *E. coli* DH5α were grown and adjusted to
263 OD_{600} = 0.1 (~5×10^6 CFU). The apical compartment was inoculated with 50 µl of each strain
264 (MOI 100 per organism). After 6 hours of incubation, media from the basal compartment was
265 collected and plated on MacConkey agar for *E. coli* and CEFEX agar for *C. jejuni*. CEFEX agar
266 was incubated at 37°C with 10% CO_2 for 48 hours; MacConkey plates were incubated aerobically
267 at 37°C for 24 hours. Each strain was tested in three independent biological experiments with two
268 technical replicates each (n = 3).
269 ***72-hour pre-infection + 3-hour E. coli exposure:*** Caco-2 BBE cells were infected with *C. jejuni*
270 as described for the TEER assay. After 72 hours, 50 µl of *E. coli* DH5α (MOI 100) was added to
271 the apical compartment and incubated for an additional 3 hours. Basolateral media was collected

272 and plated as described above. Each strain was tested in three independent biological experiments
273 with two technical replicates per experiment (n = 3). To assess intracellular *C. jejuni* and *E. coli*,
274 the apical compartment was treated with gentamicin (250 µg/ml) for 1 hour, washed, and lysed
275 with 0.1% Triton X-100. Lysates were plated on CEFEX and MacConkey agar for quantification.
276 Translocation efficiency was calculated as: (CFU in basal compartment / CFU inoculated) × 100.
277
278 **Statistical Analysis**
279 Statistical analyses were performed using GraphPad Prism (v10.4.1) for comparisons between
280 diarrheal manifestations or RStudio (v2022.07.1) for comparisons between strains. All data were
281 tested for normality (Shapiro–Wilk test) and equal variance (Levene's test). For comparisons
282 between strains associated with watery and bloody/inflammatory diarrhea (n = 15 per group), the
283 non-parametric Mann–Whitney U test was applied. For comparisons across multiple strains or
284 conditions, non-parametric Kruskal–Wallis tests with Dunn's post hoc test were used. Compact
285 Letter Display (CLD) notation was used to visualize the results of the pairwise comparisons.
286 Strains that do not share a letter are significantly different from each other ($p < 0.05$), while strains
287 sharing the same letter are not significantly different. Letters were assigned using the
288 multcompView package in R, based on adjusted p-values. Statistical significance was defined as
289 $p \leq 0.05$. Each assay included a minimum of three independent biological replicates per strain (n
290 = 3 per group), with technical replicates as noted. Data are presented as mean ± SEM unless
291 otherwise stated.
292
293
294

295 **Results**

296 ***C. jejuni strains associated with bloody/inflammatory diarrhea exhibit increased invasion and***
297 ***intracellular survival in epithelial cells.*** To assess whether *C. jejuni* strains associated with
298 distinct diarrheal phenotypes exhibit differences in virulence potential both on a strain-to-strain
299 level and between groups, we compared five strains associated with watery diarrhea and five
300 associated with bloody/inflammatory diarrhea (**Table 1**). All ten strains have previously been
301 investigated in the neonatal piglet model to confirm their diarrheal manifestations *in vivo* (**Fig 1A-**
302 **B**). Invasion assays were conducted using INT407, Caco-2 BBE and T84 cells. Significant
303 differences were observed between several strains, notably, A9a was a significantly better invader
304 of Caco-2 BBE and T84 cells than most other strains, particularly the watery diarrhea associated
305 strains (Note: 81-176 was not tested with T84 cells), whereas all five of the watery diarrhea
306 associated strains were poor invaders regardless of the cell line tested (**Fig 2A, Supplementary**
307 **Fig S1A, Supplementary Fig S2A**). Interestingly, no significant differences were observed on a
308 strain to strain level using INT407 cells. Invasion assays conducted in polarized Caco-2 BBE cell
309 revealed significantly greater invasion by bloody/inflammatory associated strains compared to
310 watery diarrhea strains ($p = 0.0014$; **Fig 2B**) Similar trends were observed using non-polarized
311 INT407 cells ($p = 0.0006$; **Supplementary Fig S1B**) and T84 cells ($p = 0.0063$; **Supplementary**
312 **Fig S2B**).

313 To evaluate whether increased invasion correlated with improved intracellular persistence,
314 we measured viable intracellular bacteria at 24 hours post-infection in T84 cells. Strain to strain
315 variation was observed among bloody/inflammatory strains M129 and A9a, where they displayed
316 significantly higher intracellular survival than most other strains (**Fig 3A**). As a group,

317 bloody/inflammatory diarrhea associated strains exhibited significantly higher intracellular
318 survival than watery diarrhea associated strains (p = 0.0007; **Fig 3B**).
319 ***Similar attachment of C. jejuni strains across intestinal cell lines.*** To determine whether
320 differences in invasion were due to upstream differences in adherence, we assessed attachment
321 efficiency after a 1-hour infection of epithelial cell lines INT407, Caco-2 BBE and T84. Few
322 significant differences were observed between strains across INT407 (**Fig 4A**), Caco-2 BBE cells
323 (**Fig 4C**) and T84 cells (**Supplementary Fig S3A**). There were no significant differences in
324 attachment between watery and bloody/inflammatory diarrhea associated strains, regardless of the
325 cell line used: INT407 cells (p = 0.20; **Fig 4B**), Caco-2 BBE (p = 0.14; **Fig 4D**), T84 cells (p =
326 0.90; **Supplementary Fig S3B**).
327
328 ***CDT production does not distinguish between watery and bloody/inflammatory strains.*** All *C.*
329 *jejuni* strains in this study produced detectable CDT activity, as measured by the HeLa cell
330 distension assay. We did not observe any strain variation in CDT production between watery and
331 bloody/inflammatory diarrhea associated strains at either 37°C or 42°C (**Supplementary Fig**
332 **S4A)**. When compared as groups at 37°C, mean CDT titers were comparable between watery
333 (geometric mean = 0.094) and bloody/inflammatory diarrhea associated strains (geometric mean
334 = 0.086; p = 0.74; **Supplementary Fig S4B**). Due to the commensal relationship between poultry
335 and *C. jejuni*, CDT production was also measured at the poultry internal body temperature of 42°C
336 and here mean CDT titers were different between watery (geometric mean = 0.10) and
337 bloody/inflammatory strains but not statistically significant (geometric mean = 0.041; p = 0.086;
338 **Supplementary Fig S4B**).
339

340 ***Bloody/inflammatory strains exhibit greater 24-hour intracellular survival in T84 cells but***
341 ***induce similar IL-8 responses.*** To evaluate whether differences in intracellular persistence
342 affected epithelial inflammatory signaling, we quantified human IL-8 production in T84 cell
343 supernatants at 24 hours post-infection and measured viable intracellular *C. jejuni* following
344 gentamicin treatment and lysis. IL-8 production was not significantly different between strains but
345 did show variability (**Supplementary Fig S5A**). Strain A9a induced the highest mean IL-8 levels
346 (1202.04 pg/ml), whereas D33a produced the lowest (437.03 pg/ml), highlighting strain-level
347 variability. Strains associated with bloody/inflammatory diarrhea demonstrated significantly
348 greater intracellular survival at 24 hours than watery diarrhea strains ($p = 0.0007$; **Fig 3B**). Despite
349 this, IL-8 secretion was not significantly different between groups: mean concentrations were
350 809.10 pg/ml for watery strains and 702.60 pg/ml for bloody/inflammatory strains ($p = 0.61$;
351 **Supplementary Fig S5B**).
352
353 ***Bloody/inflammatory strains more profoundly disrupt epithelial barrier function over time.*** To
354 assess whether *C. jejuni* strains differ in their capacity to disrupt epithelial barrier integrity, we
355 measured TEER in polarized Caco-2 BBE monolayers at 24-, 48-, and 72-hours post-infection.
356 Across 24-, 48- and 72-hours, strains A14a, A13a and S3 had the lowest changes in TEER while
357 strains A9a, M129 and 81-176 had the highest changes in TEER (**Fig 5A**). When grouped by
358 diarrheal manifestation, bloody/inflammatory diarrhea associated strains caused a significantly
359 greater reduction in TEER at all time points. At 24 hours, TEER decreased by an average 15.9%
360 compared to 4.03% in watery diarrhea associated strains ($p < 0.0001$; **Fig 5B**). Differences
361 remained significant at 48 hours (27.1% verses 10.7%; $p < 0.0001$) and 72 hours (43.4% verses
362 18.3%; $p < 0.0001$).

363

364 ***Bloody/inflammatory strains increase paracellular permeability to small molecules.*** To directly
365 measure functional barrier permeability, we quantified apical-to-basolateral flux of 4-kDa FITC-
366 dextran across polarized Caco-2 BBE monolayers following 72-hour infection. Strain-specific
367 permeability values were observed to be highest among strain A9a (a bloody/inflammatory strain)
368 while the lowest was observed among strain A13a (a watery diarrhea associated strain; **Fig 6A**).
369 Infection with bloody/inflammatory strains resulted in a 2-fold higher FITC-dextran translocation
370 compared to watery strains ($p = 0.0027$; **Fig 6B**).

371

372 ***C. jejuni promotes translocation of commensal E. coli across the epithelial barrier.*** We next
373 assessed whether barrier disruption by *C. jejuni* strains altered host susceptibility to microbial
374 translocation. Using polarized Caco-2 BBE monolayers in Transwell inserts, we evaluated whether
375 a non-invasive commensal strain of *E. coli* (DH5α) could cross the epithelial barrier during or after
376 infection with *C. jejuni* strains. Two experimental approaches were tested: (i) co-inoculation of *C.*
377 *jejuni* and *E. coli* for 6 hours, and (ii) pre-infection with *C. jejuni* for 72 hours followed by 3-hour
378 exposure to *E. coli*. In both models, *E. coli* translocation was observed among all strains and there
379 were no significant differences between strains (**Supplementary Fig S6A, S6C**). During co-
380 inoculation, the mean *E. coli* CFU recovered from the basal chamber was not significantly different
381 between bloody/inflammatory and watery diarrhea associated strains ($p = 0.66$; **Supplementary**
382 **Fig S6B**). In the sequential infection model, basal recovery of *E. coli* was more pronounced, but
383 no significant difference was observed between bloody/inflammatory and watery diarrhea
384 associated strains ($p = 0.30$; **Supplementary Fig S6D**).

385 ***Strains associated with bloody/inflammatory diarrhea demonstrate enhanced intracellular***
386 ***survival in macrophages.*** To investigate whether there was strain variation in the interactions with
387 innate immune cells, we measured intracellular survival of the *C. jejuni* strains in J774A.1 murine
388 macrophages at 24-, 48-, and 72-hours post-infection. There were significant differences between
389 strains at all time points. Notably, strain A9a had the highest percent survivability across all time
390 points while 81-176 had the lowest (**Fig 7A, 7C, 7E**). At 24 hours, viable intracellular *C. jejuni*
391 was 2.6-fold higher among bloody/inflammatory diarrhea associated strains but there was no
392 significant difference compared to watery diarrhea associated strains ($p = 0.23$; **Fig 7B**). At 48
393 hours post infection, bloody/inflammatory diarrhea associated strains showed significantly greater
394 intracellular survivability ($p = 0.011$; **Fig 7D**). However, by 72-hours post infection, there was
395 again no significant difference between bloody/inflammatory and watery diarrhea associated
396 strains ($p = 0.43$; **Fig 7F**).
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408 **Discussion**

409 Clinical outcomes associated with different *C. jejuni* strains vary widely, from mild, self-
410 limiting watery diarrhea to more severe presentations involving bloody and/or inflammatory
411 diarrhea, abdominal cramping, and fever[10]. While host factors such as immune status and
412 microbiota composition influence disease severity, accumulating evidence suggests that bacterial
413 strain differences also contribute significantly to clinical heterogeneity[6, 32]. However,
414 identifying phenotypic or molecular markers that distinguish high- and low-virulence *C. jejuni*
415 strains has proven challenging[33]. While group-level differences were clear, we observed notable
416 variation within each group, consistent with strain-to-strain variations from studies mentioned
417 previously. Some watery strains exhibited moderate invasion or barrier disruption, and not all
418 bloody/inflammatory strains displayed the most extreme phenotypes. A study by Law et al
419 performed invasion, attachment and macrophage survivability assays using six out of the 10 *C.*
420 *jejuni* strains used in this study[21]. In contrast to our study which used human intestinal epithelial
421 cells (Caco-2 BBE, INT-407 and T84), the previous study used non-polarized human epithelial
422 cells (HEp-2) that don't form tight junctions for attachment and invasion assays. Our study has
423 shown that different cell lines can provide very different results among *C. jejuni* strains.
424 Furthermore, macrophage survivability was only performed at 72 hours post infection, missing
425 significant differences at 48 hours that exist between watery and bloody/inflammatory diarrhea
426 associated strains. This study dramatically expands from the Law et al study by examining a more
427 detailed phenotypic analysis between *C. jejuni* strains, in addition to approaching the strains as
428 two groups with distinct diarrheal manifestations confirmed in neonatal piglets.

429 Using a neonatal piglet model, which closely recapitulates human *C. jejuni* associated
430 diarrheal disease[21, 29, 34], *C. jejuni* strains can be classified into two groups that consistently

431 induce a particular diarrheal manifestation of either watery diarrhea or bloody/inflammatory in the
432 neonatal piglet model[21, 34, 35]. The strains used in this study were chosen for their known
433 diarrheal manifestation as well as their varied source background of either clinical or poultry
434 sources. Given that poultry are the primary reservoirs of *C. jejuni*, our strains represent both mild
435 and severe infections that can arise from poultry. Individuals experiencing milder, self-limiting
436 symptoms such as watery diarrhea may be less likely to seek medical attention, leading to a lack
437 of clinical isolates. By comparing *in vitro* virulence traits, we found significant strain to strain
438 variation both within and between diarrheal manifestation groups, particularly among strains
439 associated with bloody/inflammatory diarrhea which consistently exhibited enhanced invasion,
440 intracellular persistence, epithelial barrier disruption and macrophage survivability, each a
441 hallmark of a more invasive and inflammatory disease mechanism.

442 Adherence to intestinal epithelial cells is one of the primary steps to initiating *C. jejuni*
443 infection[36, 37]. Here we found no significant differences in adherence levels between watery
444 and bloody/inflammatory diarrhea associated strains to small intestinal INT407 cells or polarized
445 large intestinal cells Caco-2 BBE and T84. Non-polarized cells such as INT407 cells lack a defined
446 brush border and other features such as tight junctions that are more representative of the epithelial
447 cells of the intestinal tract[38]. While as a group adherence levels were not significantly different,
448 there was strain to strain variation observed, particularly with strain M129 having higher adherence
449 levels than strains D33a, A9a, NCTC11168, and RM1221. Other studies have observed higher
450 INT407 adherence among *C. jejuni* strains associated with febrile diarrhea or those from clinical
451 sources compared to strains from patients without diarrhea/fever or from environmental
452 sources[39, 40]. Similarly, adherence levels were not significantly different between groups when
453 using Caco-2 BBE cells, a differentiated epithelial cell line originating from the human colon[41].

454 Using this cell line, there was lower strain to strain variation, where only 81-176 had significantly
455 lower levels of adherence than strain A9a. Polarized cells such as Caco-2 BBE cells are able to
456 form polarized monolayers with microvilli, and tight junctions, in addition to displaying brush
457 border proteins that, despite being of colonic origin, are characteristic of small intestinal villus
458 cells, including villin, fimbrin and the terminal web proteins fodrin and myosin II[42-45].
459 Adherence to T84 cells was similar across all *C. jejuni* strains and no significant differences were
460 observed between diarrheal manifestation groups or between strains. T84 cells are derived from a
461 human colonic carcinoma and are highly polarized with short microvilli, tight junctions and
462 desmosomes between cells[46]. T84 cells offer a more accurate model of colonocytes due to their
463 retention of key colonic features, including high expression of colonic markers and the presence
464 of short microvilli characteristic of colonocytes[47]. Variation in the adherence of *C. jejuni* strains
465 among polarized and non-polarized tissue culture cell lines requires more investigations into the
466 mechanisms driving this individual strain variation. However, our findings support that both
467 watery and bloody/inflammatory diarrhea associated strains are able to attach at similar levels to
468 intestinal epithelial cells from the small and large intestine, however their invasiveness once at that
469 epithelial surface is where they really differ.

470 Invasion has long been regarded as a hallmark of *C. jejuni* pathogenesis and has been
471 studied extensively in tissue culture models[37, 48-51]. In the present study we found that
472 bloody/inflammatory diarrhea associated strains demonstrated significantly higher invasiveness of
473 both non-polarized and polarized intestinal epithelial cells. While the individual invasiveness of
474 bloody/inflammatory diarrhea strains varied, there were few significant differences between
475 strains from the same diarrheal manifestation, as all bloody/inflammatory diarrhea associated
476 strains were invasive and all watery diarrhea associated strains were poor to non-invasive. Several

477 groups have also observed variation in the invasiveness of *C. jejuni* strains. Everest et al (1992)
478 found that *C. jejuni* clinical isolates from patients with colitis were more invasive than isolates
479 from patients with non-inflammatory diarrhea[52]. Strain dependent invasiveness has also been
480 observed using environmental strains compared to clinical strains, where clinical strains were
481 found to be significantly more invasive[40]. Even intra-strain invasiveness has been found,
482 particularly among continuously passed laboratory strains. For example, the clinical strain 81-176
483 has been reported to range in its invasiveness from 0.8 to 1.8%[53-55], while the genome
484 sequenced clinical strain NCTC11168 (NCTC11168-GS) had diminished invasiveness and
485 translocation abilities compared to the original isolate (NCTC11168-O), highlighting the potential
486 effects of high laboratory passage on strain pathogenicity and colonization[56]. Furthermore,
487 Cooper et al (2013) found that NCTC11168-GS had decreased disease production in the neonatal
488 piglet model compared to NCTC11168-O[35]. Taken together, the consistent invasiveness
489 observed among bloody/inflammatory diarrhea associated strains in our study, regardless of cell
490 line used, supports invasion as a defining characteristic of the bloody/inflammatory diarrheal
491 manifestation. The lack of invasiveness among watery diarrhea-associated strains may reflect
492 alternative pathogenic strategies, such as growth capabilities and metabolic adaptability, that do
493 not rely on epithelial invasion. These differences in invasive capacity likely contribute to the
494 distinct clinical presentations observed between strain groups and reinforce the need for broader
495 strain inclusion in *C. jejuni* pathogenesis research.

496 Bloody/inflammatory strains not only exhibit greater invasion of both polarized and non-
497 polarized epithelial cells but also demonstrate enhanced intracellular persistence. Intracellular
498 survival of *C. jejuni* has been linked to proinflammatory signaling and immune evasion in both *in*
499 *vitro* and *in vivo* models [31, 57], but this is the first study to demonstrate that such phenotypes are

500 heightened in strains associated with a consistent bloody/inflammatory diarrheal manifestation *in*
501 *vivo*. Through macrophage survivability assays, we observed a connection to immune evasion by
502 bloody/inflammatory diarrhea associated strains where these strains exhibited prolonged
503 persistence within macrophages up to 48 hours post-infection. This suggests that not only are
504 bloody/inflammatory diarrhea associated strains able to survive for at least 24 hours within
505 intestinal epithelial cells, but they also have an ability to evade phagocytic killing for several days.
506 Strain variation in macrophage survivability was most pronounced between bloody/inflammatory
507 diarrhea strains, particularly among strain A9a at 24 hours where it had higher levels of
508 survivability, although not statistically significant, compared to all other strains. Consistent with
509 our study, Day et al (2000) and Law et al (2009) found that M129 was able to be recovered from
510 J774A.1 macrophages after 72-hours, while the later study also observed survivability among A9a,
511 A13a, A14a and D42a[21, 58]. Survivability differences between watery and bloody/inflammatory
512 diarrhea associated strains at 48 hours, but not at 24 or 72 hours, suggests a transient but critical
513 window during which certain *C. jejuni* strains, particularly those associated with
514 bloody/inflammatory diarrhea, are better equipped or contain alternative mechanisms to persist
515 slightly longer within host immune cells. *C. jejuni* contains one catalase enzyme, encoded by the
516 *katA* gene, which aids in protection from reactive oxygen species generated during the oxidative
517 burst once the bacterium is phagocytosed within macrophages[59, 60]. The *katA* gene has been
518 found to be critical for *C. jejuni* macrophage survivability, where wildtype strain M129 was shown
519 to be able to persist at levels significantly higher than a M129 *katA* mutant at 24, 48 and 72 hours
520 within J774A.1 macrophages[58]. Another study found that the downregulation of *katA* in *C. jejuni*
521 strain NCTC11168 through a mutation in a MarR-type transcriptional regulator (Cj1556) was also
522 reduced in J774A.1 macrophage survivability[61, 62]. Future studies should investigate catalase

523 expression or activity contributes to the prolonged intracellular persistence observed in
524 bloody/inflammatory diarrhea-associated strains.

525 Disruption of epithelial barrier function further differentiated the two diarrheal
526 manifestation groups. Bloody/inflammatory strains induced greater reductions in TEER and
527 increased paracellular flux of FITC-dextran across polarized monolayers - both indicators of tight
528 junction impairment[63, 64]. Other studies evaluating the changes to the TEER *in vitro* utilizing
529 invasive *C. jejuni* strains, particularly 81-176 and NCTC11168, did find a significant drop in the
530 TEER. However, none of these studies utilized strains with defined diarrheal manifestations or
531 compared them to watery diarrhea associated strains[65-68]. This disruption in barrier integrity
532 was significantly more pronounced in bloody/inflammatory strains and our study is the first to
533 directly link this phenotype with reproducible diarrheal manifestations across multiple isolates.
534 The effects we observed on the integrity of the Caco-2 BBE monolayers, regardless of defined
535 diarrheal manifestation, translated into increased translocation of a non-invasive commensal *E.*
536 *coli* strain, providing evidence that the barrier compromise facilitated microbial movement across
537 the epithelium. The translocation of noninvasive bacteria such as *E. coli* during *C. jejuni* infection
538 has been previously investigated *in vitro* with varying results. Konkel et al (1992) observed no
539 change in the TEER or translocation of *E. coli* DH5α up to 6 hours following inoculation of Caco-
540 2 cell monolayers using bloody/inflammatory strain M129 and strains of unknown diarrheal
541 manifestations; F38011, 81116 and 78-27[69]. In our study, despite watery diarrhea associated
542 strains being poorly invasive or non-invasive, we observed *E. coli* translocation at similar levels
543 to bloody/inflammatory strains, suggesting alternate means for *E. coli* translocation other than
544 invasion. Translocation of *E. coli* was found to not correspond with *C. jejuni* invasiveness in one
545 study that used invasion-defective *C. jejuni* flagella mutant strains, with lipid raft-mediated

546 endocytosis suggested to induce this effect[70]. The use of M cells to promote transcytosis of non-
547 invasive bacteria was also observed using bloody/inflammatory diarrhea associated strain 81-176
548 but was not investigated using any other strains[71]. Both lipid rafts and M cells should be further
549 investigated as potential mechanisms behind *E. coli* translocation by invasive and non-invasive *C.*
550 *jejuni* strains.

551 Not all measured pathogenicity phenotypes differed between the two manifestation groups.
552 Our study found that CDT production levels were not altered both between strains and across
553 diarrheal manifestation groups at either temperature, suggesting CDT is not a discriminating factor
554 for diarrheal severity. CDT is widespread among *C. jejuni* isolates and contributes to host cell
555 cycle arrest, DNA damage, and epithelial disruption[72], however the specific contributions of
556 CDT in *C. jejuni* pathogenesis remains unclear[73, 74]. There is limited evidence that CDT
557 expression is critical to disease but it was found that CDT contributes to greater adherence and
558 invasion of epithelial cells along with more progressive gastritis and proximal duodenitis in two
559 *in vivo* mouse models[75, 76]. However, the role of CDT in driving different diarrheal symptoms
560 is not well understood. Previous studies have assessed CDT production with HeLa cells among *C.*
561 *jejuni* strains from clinical or poultry sources and have reported variability, although none had a
562 diarrheal manifestation associated with any of the strains[77-79]. Additionally, IL-8 secretion, a
563 hallmark of initial epithelial response to *C. jejuni* contact[80-82] and a potent chemoattractant and
564 cellular activator[81] also did not differ significantly between diarrheal manifestation groups at 24
565 hours post-infection. Although not statistically significant, strain to strain variation was observed
566 among bloody/inflammatory strains with D33a and NCTC11168 having lower IL-8 levels than
567 M129 and A9a. Watery diarrhea associated strains showed less variation between strains with only
568 A13a and A14a having lower IL-8 levels than other watery diarrhea associated strains. Few studies

569 have looked at differences in IL-8 secretion across *C. jejuni* strains and this is the first study to
570 assess IL-8 secretion by *C. jejuni* strains with known diarrheal manifestations in the neonatal piglet
571 model. One study using bloody/inflammatory diarrhea associated strain NCTC11168 and human
572 colitis strain L115 showed no difference in IL-8 secretion by T84 cells[83], while another study
573 looked at IL-8 secretion between *C. jejuni* strains from poultry and human sources and found no
574 difference in IL-8 secretion by T84 cells between groups[84]. However, IL-8 secretion is not based
575 solely on the invasive or adherent capability of *C. jejuni* strains, as it can also be activated by CDT
576 production[81, 82], indicating that early chemokine responses may not correlate with longer-term
577 invasive or inflammatory phenotypes. Furthermore, our findings that both bloody/inflammatory
578 and watery diarrhea associated strains produce similar levels of CDT and elicit comparable IL-8
579 responses despite significant differences in invasion. This variation highlights the importance of
580 considering strain-level differences when evaluating the inflammatory potential of *C. jejuni* and
581 suggests that multiple mechanisms may drive IL-8 secretion in the host.

582 Strain A9a, originally recovered from a poultry processing plant in Kansas, USA[85], is
583 associated with bloody/inflammatory diarrhea in the neonatal piglet model. A9a demonstrated the
584 highest levels of adherence, invasion, and intracellular survival to intestinal epithelial cells
585 compared to all other *C. jejuni* strains. While its CDT production was comparable to many of the
586 other *C. jejuni* strains, A9a showed the highest stimulation of IL-8 secretion from T84 cells, results
587 that support IL-8 production due to high adherence and invasion even with similar levels of CDT.
588 A9a also caused the most pronounced disruption to epithelial barrier integrity, with the steepest
589 decline in TEER, the highest FITC-dextran permeability, and the greatest translocation of *E. coli*
590 DH5α across Caco-2 BBE monolayers over 72 hours. Finally, A9a had the highest survivability
591 within macrophages across all time points, reflecting its resilience against innate immune

592 responses. Despite its strong *in vitro* virulence potential, A9a was not hypervirulent in the piglet
593 model[21]. Instead, it produced bloody/inflammatory diarrhea at similar levels to the other strains
594 in this study, 81-176, M129, D33a and NCTC11168, suggesting that multiple factors may drive
595 the severity of diarrhea. *C. jejuni* strain variation has been observed in other studies, such as Ex114,
596 a *C. jejuni* strain that was found to be 25 times more invasive than reference strain 81116, which
597 is a relatively low invader[16]. Consistent with our findings, the same study reported that increased
598 invasiveness was not linked to enhanced adhesion, and motility levels were similar between highly
599 and minimally invasive strains. In other studies, stain 81-176, sourced from a raw milk
600 outbreak,[86] is widely regarded as a highly invasive clinical isolate and has been shown to cause
601 robust epithelial invasion in cell lines[53, 87] and animal models[88] as well as produce disease
602 in humans[89]. Yet its performance varies between laboratories depending on passage history[53,
603 55, 90]. Repeatedly passages reduced the invasiveness and serum survival of 81-176[91].
604 Similarly, while strain NCTC11168 is a reference strain in *C. jejuni* research, its virulence is
605 known to diminish with laboratory passage, leading to reduced motility, invasion and colonization
606 capacity *in vitro*[56] in addition to altering *in vivo* virulence in the neonatal piglet model[35]. Our
607 findings support these previous reports that strain-dependent variation plays a key role in
608 pathogenicity.

609 This strain-to-strain variations observed in this study, even among strains associated with
610 a particular diarrheal manifestation in the neonatal piglet model, highlight the inherent complexity
611 and plasticity of *C. jejuni* virulence[92-95]. This study also underscores the need for population-
612 scale studies integrating phenotypic data with comparative genomics to identify combinations of
613 traits that drive disease severity. One such study that performed a comparative genomic analysis
614 on 67 *C. jejuni* and 12 *C. coli* strains, identified the *Campylobacter jejuni*-integrated elements

615 (CJIEs) originally identified in *C. jejuni* strain RM1221, were variably present across other
616 *Campylobacter* strains[96]. These genomic elements, some of which included prophages and
617 mobile genetic elements, contribute to functional differences that may affect virulence, stress
618 response, or host interaction. Notably, Parker et al reported that coding sequences within CJIEs
619 were highly variable, highlighting a potential genomic mechanism for the phenotypic diversity
620 observed in our assays[96]. This supports our findings that even within clinically defined groups,
621 virulence phenotypes can differ, potentially due to underlying genomic heterogeneity. One
622 limitation of this study is that the number of strains included was limited, but they were well-
623 characterized and associated with consistent clinical phenotypes in a validated *in vivo* neonatal
624 piglet model. Our *in vitro* assays cannot fully replicate host complexity, but the consistency of
625 results across multiple models strengthens confidence in the observed trends. Future work should
626 integrate whole-genome sequencing, transcriptomic profiling, and *in vivo* validation to identify the
627 molecular determinants responsible for these virulence phenotypes.

628 In summary, *C. jejuni* strains associated with bloody/inflammatory diarrhea exhibit a
629 distinct virulence profile characterized by epithelial invasion, intracellular survival, barrier
630 disruption, and macrophage persistence (**Fig 8**). Together, enhanced invasion and intestinal
631 damage, intracellular survival, and macrophage survivability provide a functional explanation for
632 the more severe pathology observed in the piglet model by bloody/inflammatory strain and, by
633 extension, potentially in human infections. By linking *in vivo* disease phenotypes to mechanistic
634 *in vitro* traits, our findings advance understanding of *C. jejuni* pathogenesis and provide a
635 framework for developing targeted diagnostics, risk-based screening tools, and novel therapeutic
636 interventions. Furthermore, the strain-to-strain variation observed in this study, even within groups

637 associated with the same *in vivo* diarrheal manifestation, present that caution is needed when
638 interpreting the results of experiments that utilize only a few *C. jejuni* strains.
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Ethics statement

This study did not involve the use of animals or human participants. The *Campylobacter jejuni* strains used in this study were previously characterized in a neonatal piglet model under approved animal use protocols, as described in Law et al and Cooper et al. No new *in vivo* experiments were conducted for this study.

Data availability statement

All relevant data are within the manuscript and its supplementary files.

Author Contributions

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.

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Competing interests

The authors declare no competing interests.

672 **Tables**

673 **Table 1.** *Campylobacter jejuni* strains utilized in this study

Strain	Source	Sequence Type (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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690 **Figure Legends**

691 **Fig 1. Gross pathology associated with different diarrheal outcome caused by different**
692 **strains of *Campylobacter jejuni* based on previous challenge in colostrum deprived neonatal**
693 **piglet model.** (A) Gross pathology of the intestinal tract of neonatal piglets infected with *C. jejuni*
694 strains associated with watery diarrhea manifestation. (B) Gross pathology of the intestinal tract
695 of neonatal piglets infected with *C. jejuni* strains associated with bloody and/or inflammatory
696 diarrhea manifestation. Piglet studies were conducted prior to this study and were described
697 previously in Law et al and Cooper et al[21, 35].

698

699 **Fig 2. Average percent invasion of intestinal epithelial cell line Caco-2 BBE.** (A) Invasion
700 across all ten *C. jejuni* strains. Bars labeled with different letters indicate statistically significant
701 differences ($p < 0.05$) based on Dunn's post hoc test following a Kruskal-Wallis analysis. Shared
702 letters denote groups that are not significantly different from each other. (B) Invasiveness of Caco-
703 2 BBE cells by *C. jejuni* strains grouped by diarrheal manifestation (**, $p = 0.0014$).

704

705 **Fig 3. Intracellular survival over 24 hours in T84 cells.** (A) Average percent survival within
706 T84 cells by strain. Bars labeled with different letters indicate statistically significant differences
707 ($p < 0.05$) based on Dunn's post hoc test following a Kruskal-Wallis analysis ($n = 3$). Shared letters
708 denote groups that are not significantly different from each other. (B) Percent T84 24-hour
709 intracellular survival by *C. jejuni* strains grouped by diarrheal manifestation (***, $p = 0.0007$).
710 Strain 81-176 is not included in this data set.

711

712 **Fig 4. Adherence to intestinal epithelial cell lines.** (A) Adherence of each *C. jejuni* strain to
713 INT407 cells. Bars labeled with different letters indicate statistically significant differences ($p <$
714 0.05) based on Dunn's post hoc test following a Kruskal-Wallis analysis ($n = 3$). Shared letters
715 denote groups that are not significantly different from each other. (B) Adherence to INT407 cell
716 by *C. jejuni* strains grouped by diarrheal manifestation ($p = 0.20$). (C) Adherence of each *C. jejuni*
717 strain to Caco-2 BBE cells ($n = 3$). (D) Adherence to Caco-2 BBE cells by *C. jejuni* strains grouped
718 by diarrheal manifestation ($p = 0.14$).
719
720 **Fig 5. Change in the transepithelial electrical resistance (TEER) over 24, 48 and 72 hours.**
721 (A) Change in TEER for each *C. jejuni* strain over 24-, 48- and 72-hours. Those labeled with
722 different letters indicate statistically significant differences ($p < 0.05$) based on Dunn's post hoc
723 test following a Kruskal-Wallis analysis ($n = 3$). Shared letters denote groups that are not
724 significantly different from each other. (B) Change in TEER across *C. jejuni* strains grouped by
725 diarrheal manifestation at 24 hours (****, $p < 0.0001$), 48 hours (****, $p < 0.0001$), and at 72
726 hours (****, $p < 0.0001$). Mock infection represents Caco-2 BBE cells that contained only sterile
727 cell culture media.
728
729 **Fig 6. The fluorescence of FITC-Dextran flow through after 72 hours of infection with *C.***
730 ***jejuni* strains.** (A) Average FITC-Dextran fluorescence intensity of each *C. jejuni* strain. Bars
731 labeled with different letters indicate statistically significant differences ($p < 0.05$) based on
732 Dunn's post hoc test following a Kruskal-Wallis analysis ($n = 3$). Shared letters denote groups that
733 are not significantly different from each other. (B) There was a highly significant difference

734 between the watery and bloody/inflammatory diarrhea associated strains (**, p = 0.0027). Mock
735 infection represents Caco-2 BBE cells that contained only sterile cell culture media.
736
737 **Fig 7. Survivability of *C. jejuni* strains in J774A.1 macrophages over 24, 48 and 72 hours.** (A)
738 Survivability by each *C. jejuni* strain over 24 hours. Bars labeled with different letters indicate
739 statistically significant differences ($p < 0.05$) based on Dunn's post hoc test following a Kruskal-
740 Wallis analysis. Shared letters denote groups that are not significantly different from each other.
741 Average percent survivability was calculated based on three biologically independent assays with
742 two technical replicates (n = 3). (B) Macrophage survivability of *C. jejuni* strains grouped by
743 diarrheal manifestation over 24 hours (p = 0.23). (C) Survivability by each *C. jejuni* strain over
744 48-hours. (D) Macrophage survivability of *C. jejuni* strains grouped by diarrheal manifestation
745 over 48 hours (*, p = 0.011). (E) Survivability of *C. jejuni* strains over 72 hours. (F) Macrophage
746 survivability of *C. jejuni* strains grouped by diarrheal manifestation over 72 hours (p = 0.43).
747
748 **Fig 8. Proposed lifestyles of *C. jejuni* strains associated with watery or bloody/inflammatory**
749 **diarrhea.** Watery diarrhea associated strains live an extracellular lifestyle where they attach to
750 enterocytes and produce CDT toxin. They are poor invaders, disrupting the tight junctions between
751 epithelial cells to a minimal degree, although commensal bacterial such as *E. coli* are translocated.
752 Those that do weakly invade, do not survive long term intracellularly and are effectively removed
753 by macrophages. Bloody/inflammatory diarrhea associated strains support an intracellular lifestyle
754 where they attach to and invade enterocytes, significantly disrupting the tight junctions between
755 those enterocytes. Bloody/inflammatory diarrhea associated strains can evade killing by

756 macrophages for longer periods of time, promoting infection and contributing to more severe
757 patient symptoms. Figure created in BioRender.
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765 **Supplementary Figure Legends**
766 **Supplementary Fig S1. Invasion of INT407 cells.** (A) Invasion across all ten *C. jejuni* strains.
767 Bars labeled with different letters indicate statistically significant differences ($p < 0.05$) based on
768 Dunn's post hoc test following a Kruskal-Wallis analysis. Shared letters denote strains that are not
769 significantly different from each other. (B) Invasiveness of INT407 cells by *C. jejuni* strains
770 grouped by diarrheal manifestation (***, $p = 0.0006$).
771
772 **Supplementary Fig S2. Average percent invasion of intestinal epithelial cell line T84.** (A)
773 Invasion of T84 cells across all ten *C. jejuni* strains. Bars labeled with different letters indicate
774 statistically significant differences ($p < 0.05$) based on Dunn's post hoc test following a Kruskal-
775 Wallis analysis. Shared letters denote groups that are not significantly different from each other.
776 (B) Invasiveness of *C. jejuni* strains grouped by diarrheal manifestation (**, $p = 0.0063$).
777

778 **Supplementary Fig S3. Adherence to T84 cells.** (A) Adherence to T84 cells by *C. jejuni* strains.
779 Bars labeled with different letters indicate statistically significant differences ($p < 0.05$) based on
780 Dunn's post hoc test following a Kruskal-Wallis analysis. Shared letters denote groups that are not
781 significantly different from each other ($n = 3$). Strain 81-176 was not included in this data set. (B)
782 Adherence to T84 cells by *C. jejuni* strains grouped by diarrheal manifestation ($p = 0.90$). Strain
783 81-176 is not included in this data set.
784
785 **Supplementary Fig S4. Cytolethal Distending Toxin (CDT) production at 37°C and 42°C.**
786 (A) CDT toxin production at 37°C and 42°C by *C. jejuni* strains. (B) CDT production *C. jejuni*
787 strains grouped by diarrheal manifestation at 37°C ($p = 0.74$) and 42°C ($p = 0.086$). CDT
788 production was not significantly different when performed at 37°C compared to 42°C for watery
789 diarrhea associated strains ($p = 0.65$) or bloody/inflammatory diarrhea associated strains ($p = 0.12$).
790
791 **Supplementary Fig S5. IL-8 Production following 24-hour incubation with *C. jejuni* strains.**
792 (A) IL-8 production in pg/ml of *C. jejuni* strains. Bars labeled with different letters indicate
793 statistically significant differences ($p < 0.05$) based on Dunn's post hoc test following a Kruskal-
794 Wallis analysis. Shared letters denote groups that are not significantly different from each other.
795 Strain 81-176 is not included in this data set. Mock infection represents Caco-2 BBE cells that
796 contained only sterile cell culture media. (B) IL-8 production in pg/ml of *C. jejuni* strains grouped
797 by diarrheal manifestation ($p = 0.61$). Mock infection represents Caco-2 BBE cells that contained
798 only sterile cell culture media. Strain 81-176 is not included in this data set.
799

800 **Supplementary Fig S6. Translocation of *Escherichia coli* through Caco-2 BBE cell**
801 **monolayers.** (A) 6-hour co-culture incubation with *C. jejuni* strains. Bars labeled with different
802 letters indicate statistically significant differences ($p < 0.05$) based on Dunn's post hoc test
803 following a Kruskal-Wallis analysis. Shared letters denote groups that are not significantly
804 different from each other. (B) 6-hour co-culture incubation with *C. jejuni* strains grouped by
805 diarrheal manifestation ($p = 0.66$). Mock infection represents Caco-2 BBE cells that contained
806 only sterile cell culture media. (C) Pre-infection with *C. jejuni* for 72 hours followed by 3-hour
807 exposure to *E. coli* through Caco-2 BBE cells by strain ($n = 3$). (D) Pre-infection with *C. jejuni* for
808 72 hours followed by 3-hour exposure to *E. coli* through Caco-2 BBE grouped by diarrheal
809 manifestation ($p = 0.30$).

810

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1161

Figure 1

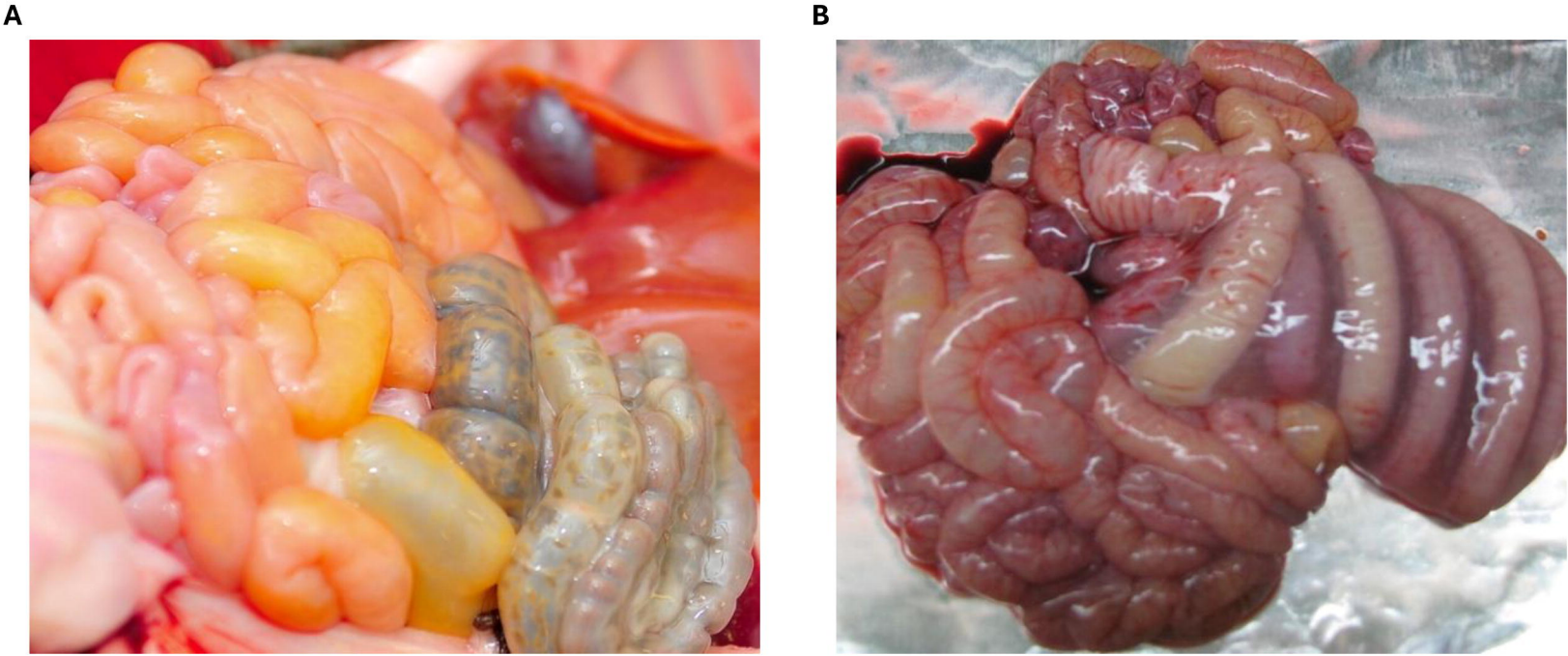
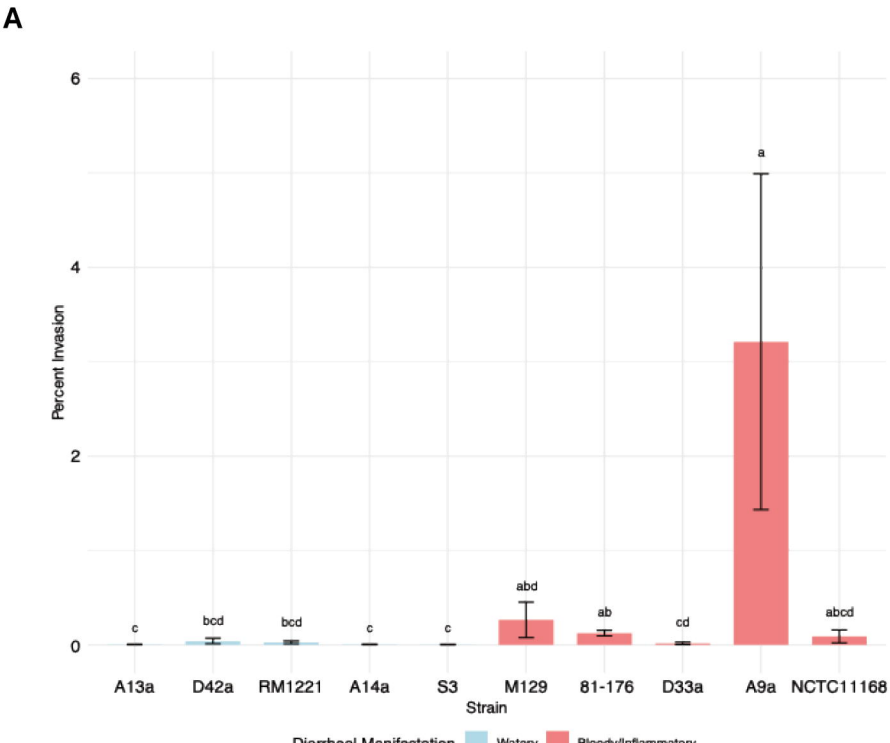


Figure 2


A
Percent Invasion
6
4
2
0
c
bcd
bcd
c
c
abd
ab
cd
a
abcd
A13a
D42a
RM1221
A14a
S3
M129
81-176
D33a
A9a
NCTC11168
Strain
Diarrheal Manifestation
Watery
Bloody/Inflammatory

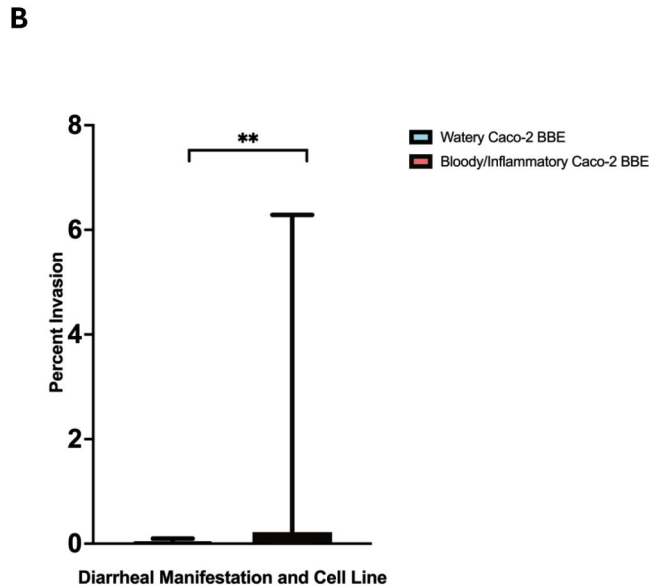
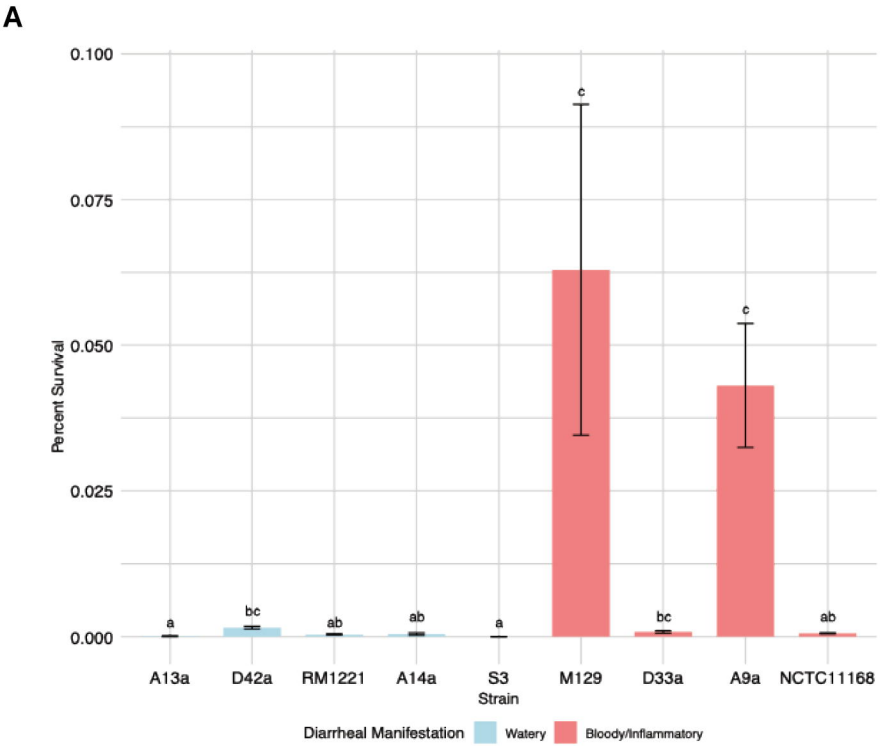

B
Percent Invasion
8
6
4
2
0
**
Watery Caco-2 BBE
Bloody/Inflammatory Caco-2 BBE
Diarrheal Manifestation and Cell Line

Figure 3


A
0.100
0.075
0.050
0.025
0.000
Percent Survival
c
c
a
bc
ab
ab
a
bc
ab
A13a
D42a
RM1221
A14a
S3
M129
D33a
A9a
NCTC11168
Strain
Diarrheal Manifestation
Watery
Bloody/Inflammatory

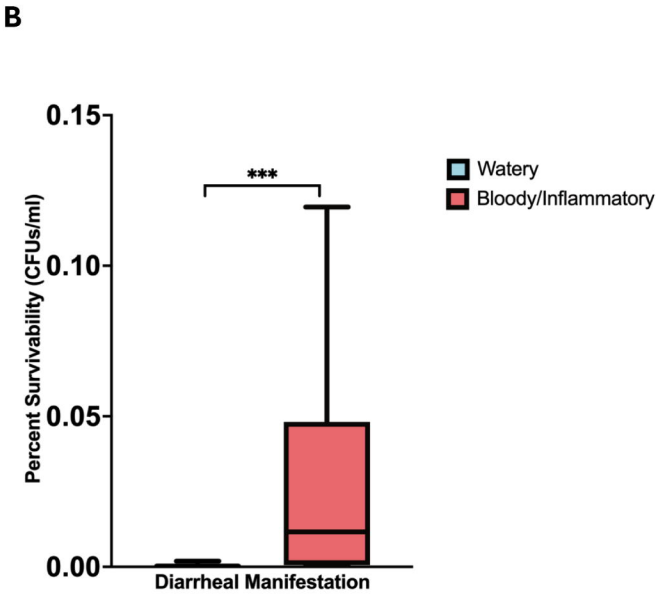

B
0.15
0.10
0.05
0.00
Percent Survivability (CFUs/ml)
***
Diarrheal Manifestation
Watery
Bloody/Inflammatory

Figure 4

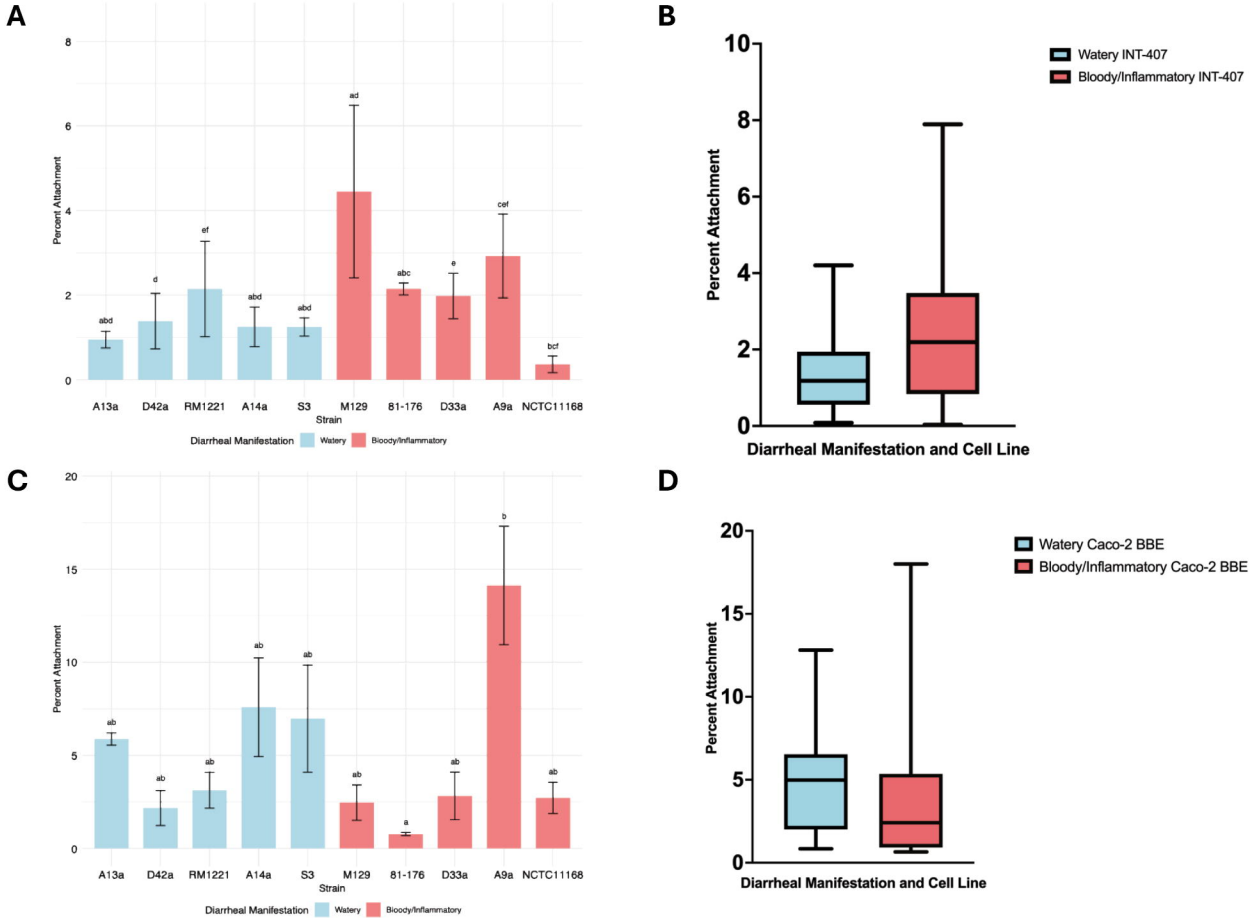

A
Percent Attachment
Strain
A13a
D42a
RM1221
A14a
S3
M129
81-176
D33a
A9a
NCTC11168
Diarrheal Manifestation
Watery
Bloody/Inflammatory
B
Watery INT-407
Bloody/Inflammatory INT-407
Percent Attachment
Diarrheal Manifestation and Cell Line
C
Percent Attachment
Strain
Diarrheal Manifestation
Watery
Bloody/Inflammatory
D
Watery Caco-2 BBE
Bloody/Inflammatory Caco-2 BBE
Percent Attachment
Diarrheal Manifestation and Cell Line

Figure 5

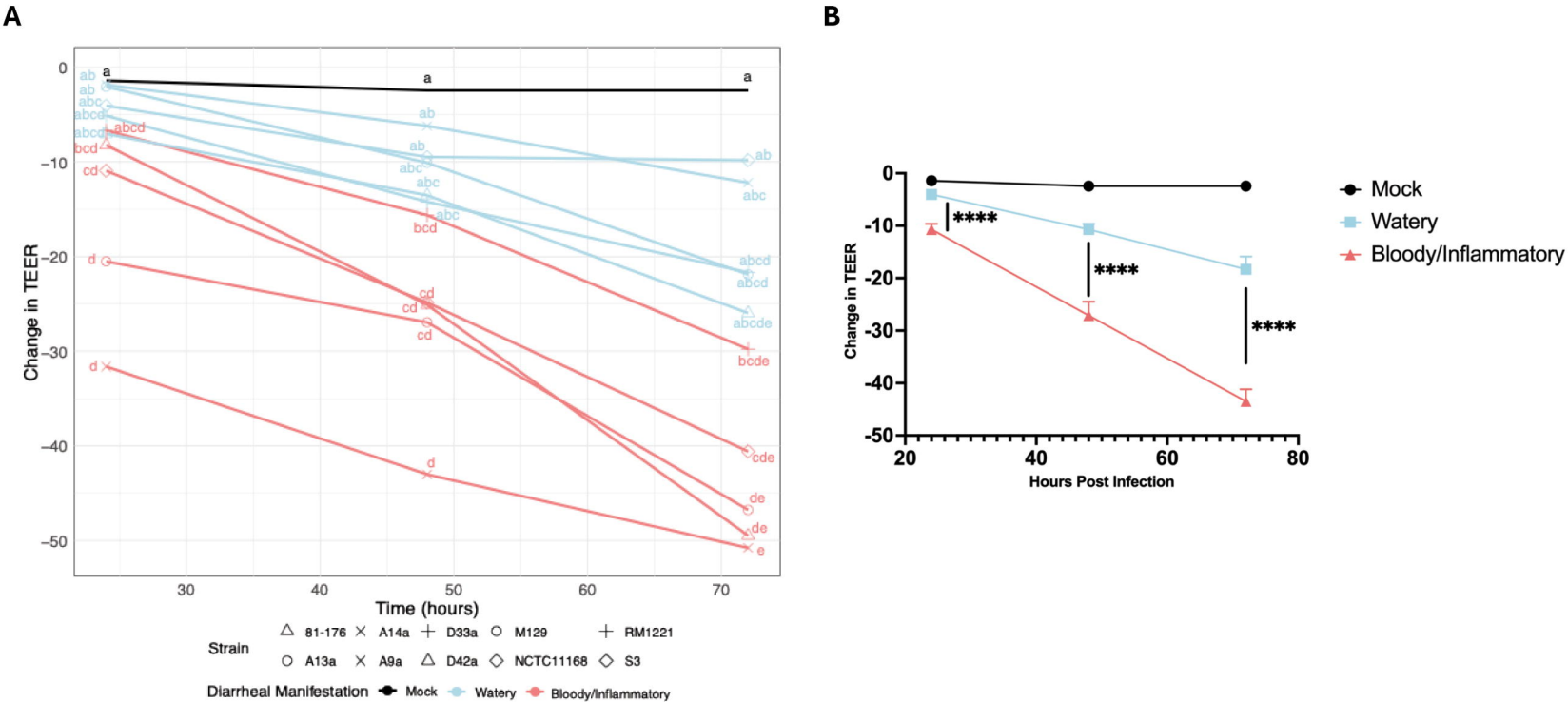
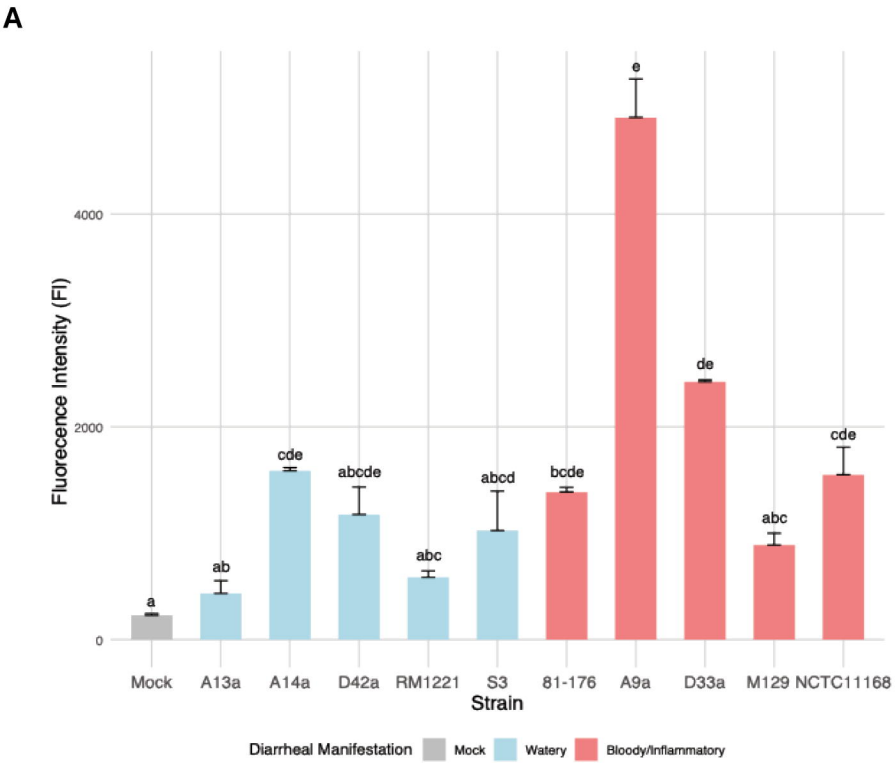

A
Change in TEER
Time (hours)
Strain
81-176
A14a
D33a
M129
RM1221
A13a
A9a
D42a
NCTC11168
S3
Diarrheal Manifestation
Mock
Watery
Bloody/Inflammatory
B
Change in TEER
Hours Post Infection
****
Mock
Watery
Bloody/Inflammatory

Figure 6


A
Fluorescence Intensity (FI)
4000
2000
0
a
ab
cde
abcde
abc
abcd
bcde
e
de
abc
cde
Mock
A13a
A14a
D42a
RM1221
S3
81-176
A9a
D33a
M129
NCTC11168
Strain
Diarrheal Manifestation
Mock
Watery
Bloody/Inflammatory

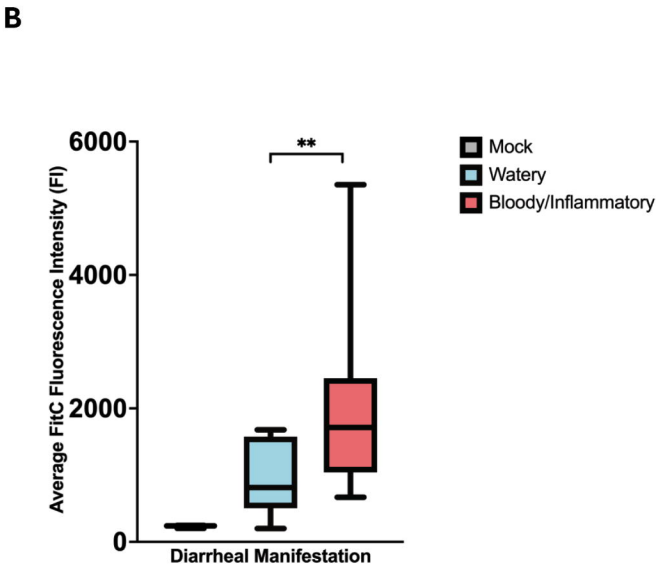

B
Average FitC Fluorescence Intensity (FI)
6000
4000
2000
0
**
Diarrheal Manifestation
Mock
Watery
Bloody/Inflammatory

Figure 7

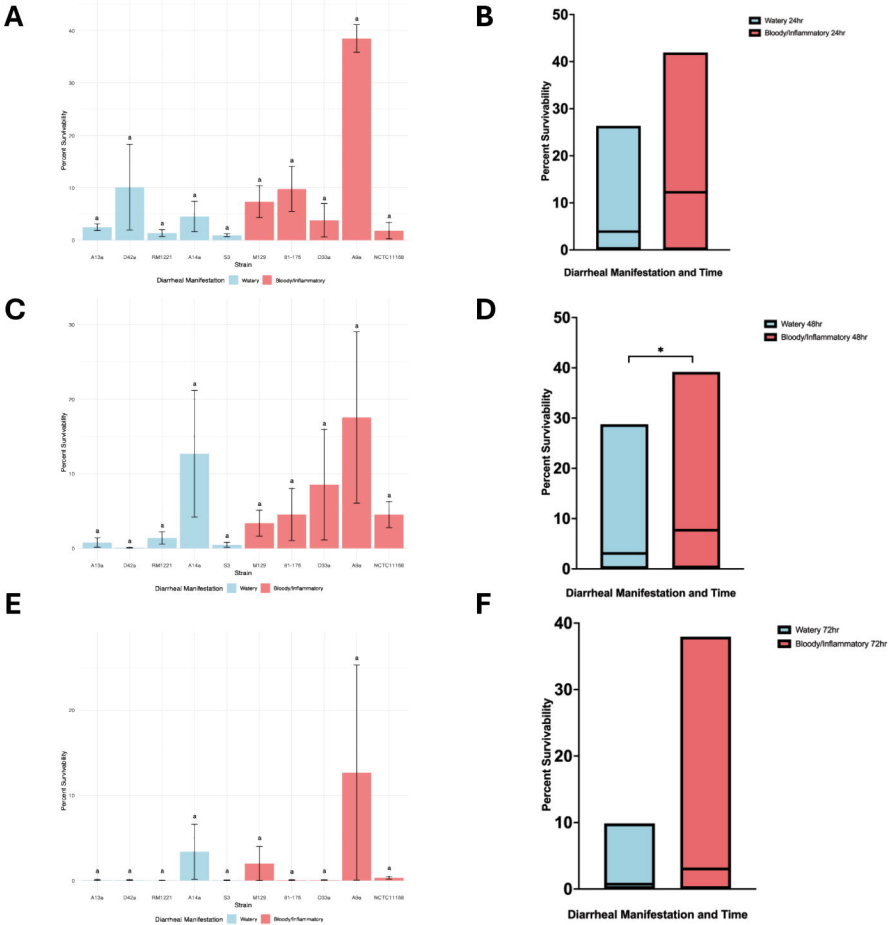
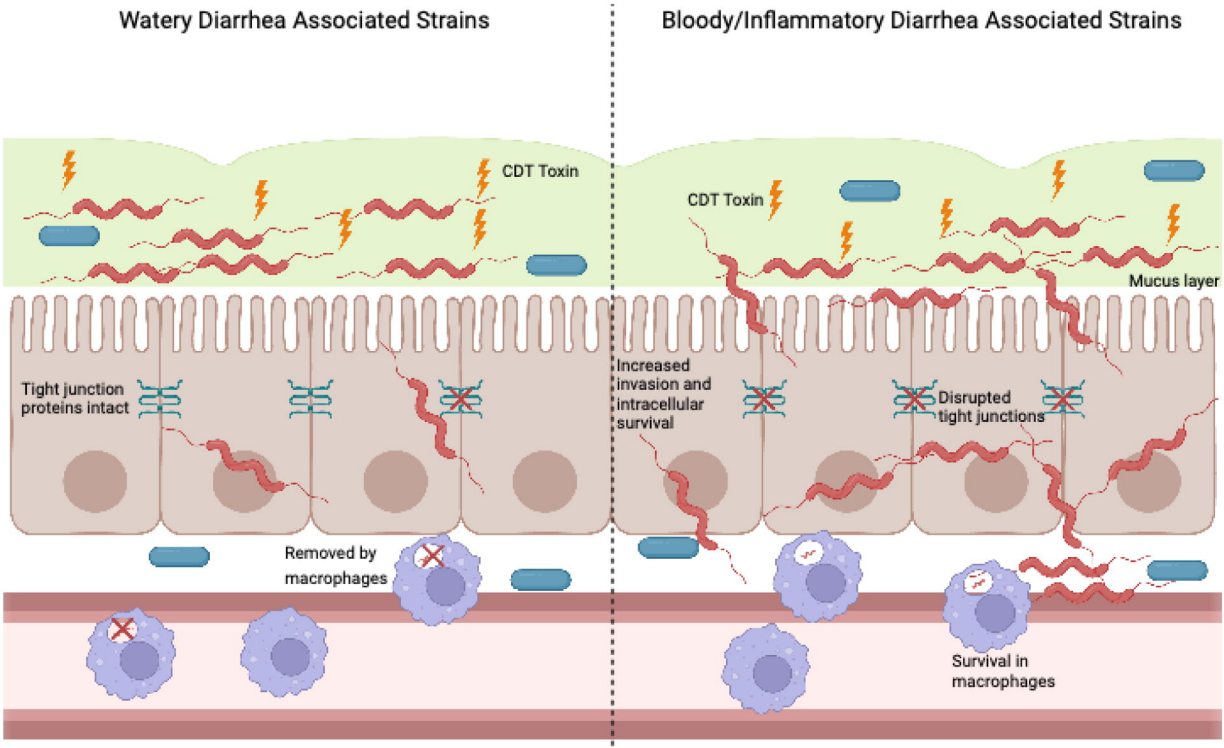

A
B
C
D
E
F
Percent Survivability
Strain
A13a
D42a
RM1221
A14a
S3
M129
81-176
D33a
A9a
NCTC11168
Diarrheal Manifestation
Watery
Bloody/Inflammatory
Watery 24hr
Bloody/Inflammatory 24hr
Watery 48hr
Bloody/Inflammatory 48hr
Watery 72hr
Bloody/Inflammatory 72hr
Diarrheal Manifestation and Time

Figure 8


Watery Diarrhea Associated Strains
Bloody/Inflammatory Diarrhea Associated Strains
CDT Toxin
CDT Toxin
Mucus layer
Tight junction proteins intact
Increased invasion and intracellular survival
Disrupted tight junctions
Removed by macrophages
Survival in macrophages