

1 **The impact of *Eimeria tenella* co-infection on *Campylobacter jejuni* colonisation**
2 **of the chicken**

3

4 ***Running title* Impact of *E. tenella* on *C. jejuni* colonisation**

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23 **Abstract**

24 *Eimeria tenella* can cause the disease coccidiosis in chickens. The direct and often
25 detrimental impact of this parasite on chicken health, welfare and productivity is well
26 recognised, however less is known about the secondary effects infection may have
27 on other gut pathogens. *Campylobacter jejuni* is the leading cause of human
28 bacterial food-borne disease in many countries and has been demonstrated to exert
29 negative effects on poultry welfare and production in some broiler lines. Previous
30 studies have shown that concurrent *Eimeria* infection can influence colonisation and
31 replication of bacteria such as *Clostridium perfringens* and *Salmonella* Typhimurium.
32 Through a series of *in vivo* co-infection experiments, this study evaluated the impact
33 that *E. tenella* infection had on *C. jejuni* colonisation of chickens, including the
34 influence of variations in parasite dose and sampling time post-bacterial challenge.
35 Co-infection with *E. tenella* resulted in a significant increase in *C. jejuni* colonisation
36 in the caeca, in a parasite dose dependent manner, but a significant decrease in *C.*
37 *jejuni* in the spleen and liver of chickens. Results were reproducible at three and ten
38 day's post-bacterial infection. This work highlights that *E. tenella* not only has a direct
39 impact on the health and well-being of chickens but can have secondary effects on
40 important zoonotic pathogens.

41 **Introduction**

42 Commercial production of chickens has increased dramatically in recent decades
43 with further expansion predicted (1, 2), increasing their relevance to human food
44 security and safety. Understanding interactions between infectious agents within the
45 chicken is important as these can influence animal welfare, commercial success and,
46 potentially, public health. Interactions within the gut are of particular importance
47 because the chicken intestinal microbiome influences performance parameters such
48 as feed conversion ratio and body weight gain (3, 4). Concurrent infections can
49 influence the colonisation and replication of pathogens in the chicken intestine, a
50 classic example being enhanced growth of *Clostridium perfringens* potentiated by
51 high mucus production induced by co-infecting *Eimeria* species parasites (5).
52 Recently, the translocation of *Escherichia coli* from the gut to internal organs was
53 shown to be enhanced by co-infection with *Campylobacter jejuni* (6). Moreover, an
54 extensive study of commercial broiler flocks showed a strong association between
55 *Campylobacter* isolation and rejection of carcasses due to unspecified microbial
56 infections (7).

57 *Eimeria tenella* and *C. jejuni* are of considerable veterinary and medical significance,
58 respectively. *Eimeria* species parasites are ubiquitous under intensive farming
59 systems (8), have a huge economic impact (9) and can affect colonisation of
60 pathogenic bacteria such as *C. perfringens* and *Salmonella enterica* Typhimurium (5,
61 10). The use of live *Eimeria* vaccines in the poultry industry and the development of
62 *Eimeria* as a vaccine vector (11, 12) prompted this investigation into the effects that
63 *Eimeria* has on other pathogenic agents found in poultry, such as *C. jejuni*.

64 *C. jejuni* is the leading cause of human bacterial food poisoning in many countries,
65 with an estimated global burden of 95 million illnesses, 21,000 deaths and 2.1 million
66 disability-adjusted life years lost in 2010 (13), and can induce severe sequelae
67 including inflammatory neuropathies such as the Guillain-Barré syndrome (14).
68 Source attribution studies unequivocally identify chickens as the major reservoir of
69 this zoonotic infection (15). *Campylobacter* is environmentally ubiquitous (16) and is
70 commonly found in and around poultry houses, with horizontal transfer being the
71 main route of infection for intensively reared broilers (15). The movement of humans
72 in and out of poultry houses appears to be extremely important in the active carriage
73 of the bacterium. Studies investigating transmission routes for *Campylobacter* on
74 farms have isolated *Campylobacter* from multiple human sources including hands,
75 boots and clothes of farm workers, drivers and managers. Molecular analysis found
76 that in numerous cases these same isolates were subsequently recovered from the
77 poultry (17). The bacterium is usually undetectable within chicken flocks during the
78 first few weeks of life and this is thought to be due to the presence of maternal anti-
79 *campylobacter* IgY antibodies which gradually decrease and disappear after two to
80 three weeks (18) (19). After this period, once the first bird becomes colonised the
81 infection spreads quickly throughout the flock via the faecal-oral route (20). *C. jejuni*
82 replicates rapidly in the intestinal mucus of chickens and transiently invades
83 epithelial cells to avoid mucosal clearance (21). Subsequently, *C. jejuni* can
84 translocate across the intestinal epithelial barrier and disseminate into deeper
85 tissues including the liver and spleen, increasing its infectious potential as internally-
86 located bacteria are less likely to be destroyed by cooking than faecal surface
87 contaminants (22). Increasingly, outbreaks of human campylobacteriosis are linked
88 to the consumption of undercooked chicken products such as liver paté (23).

89 The aim of this study was to investigate the influence of concurrent *E. tenella*
90 infection on *C. jejuni* colonisation in chickens, including investigation of physical and
91 immunological factors associated with the observed changes. *E. tenella* causes
92 haemorrhagic enteritis in the chicken caeca, accompanied by the induction of strong
93 pro-inflammatory immune responses that includes influx of heterophils, enhanced
94 mucus production, increased T-cell proliferation and a surge in the expression of a
95 variety of immune effectors (5, 24-27). We postulated that immune responses and/or
96 the pathology induced by *E. tenella* may allow *C. jejuni* to flourish and breach the
97 protective gut wall, increasing colonisation and replication within the caeca, liver and
98 spleen.

99

100 **Materials and Methods**

101 **Ethics statement**

102 The work described here was conducted in accordance with UK Home Office
103 regulations under the Animals (Scientific Procedures) Act 1986 (ASPA), with
104 protocols approved by the Institute for Animal Health and Royal Veterinary College
105 Animal Welfare and Ethical Review Bodies (AWERB). Study birds were observed
106 daily for signs of illness and/or welfare impairment and were sacrificed under Home
107 Office licence by cervical dislocation.

108 **Animals**

109 Light Sussex chickens, purchased from the Institute for Animal Health Poultry
110 Production Unit (IAH PPU, Compton, UK) were used for all experiments. All chickens
111 were certified as specific-pathogen free (SPF). Throughout the study all chickens

112 had access to food and water *ad-libitum* and were fed with a standard commercial
113 poultry grower diet including 20% protein and 55% wheat (LBS-biotech, UK).

114 **Parasites and propagation**

115 The *E. tenella* Wisconsin (Wis) strain and its derivative, the attenuated WisF96 line
116 were used throughout these studies (28, 29). The Wis strain is a wild-type (non-
117 attenuated) *E. tenella* isolate with a standard pre-patent period of ~132 hours. The
118 WisF96 line has been attenuated by selection for precocious development, resulting
119 in a single round of schizogony with a reduced pre-patent period of ~96 hours and
120 much reduced pathology due to the loss of the second generation schizont, which is
121 responsible for deep tissue damage and haemorrhage (23). Nonetheless, the
122 WisF96 line retains the ability to induce a fully protective immune response during
123 natural infection that is comparable to the non-attenuated Wis strain (28). These
124 parasites are phenotypically stable and were passaged through chickens at the
125 Institute for Animal Health, and then the Royal Veterinary College through dosing
126 and recovery as previously described (30), and used in these studies less than one
127 month after sporulation.

128 **Bacterial propagation**

129 *C. jejuni* strain 81-176 was used due to its proven ability to efficiently colonise the
130 chicken gastrointestinal tract (31). Bacteria were routinely cultured in Mueller-Hinton
131 (MH) broth and on sheep blood agar plates at 37°C for 48 hours in a microaerophilic
132 atmosphere created using the CampyGen system (all Oxoid, Basingstoke, UK).
133 Charcoal cefoperazone deoxycholate agar (CCDA, Oxoid) was used to
134 retrospectively enumerate colony-forming units of *C. jejuni* administered per animal,
135 by directly plating 10-fold serial dilutions of the inoculum in phosphate-buffered

136 saline (PBS, Oxoid). CCDA was also used to enumerate *C. jejuni* recovered from
137 chickens by directly plating 10-fold serial dilutions of homogenates of caecal
138 contents, liver and spleen (as described below). Plates were incubated at 37°C for
139 48 hours in a microaerophilic atmosphere, as detailed above. Animals not
140 challenged using *C. jejuni* were screened for exposure to *Campylobacter* by
141 enrichment of caecal contents using modified Exeter broth as described previously
142 (31) followed by plating on CCDA plates.

143 **Experimental design**

144 ***E. tenella*/*C. jejuni* co-infection**

145 Three *in vivo* trials were undertaken to investigate the influence of the presence and
146 severity of ongoing *E. tenella* infection on the outcome of oral *C. jejuni* challenge.

147 In trial 1 (pilot study, conducted at the Institute for Animal Health), 24 SPF Light
148 Sussex chickens were caged in three groups of eight. Chickens in Group 1 received
149 4,000 sporulated *E. tenella* Wis (non-attenuated, n) oocysts by oral gavage at 13
150 days of age (nE+). Chickens in Group 2 received 115,000 sporulated WisF96
151 (attenuated; a) oocysts by oral gavage at 15 days of age (aE+). Chickens in Group 3
152 were not infected with *E. tenella* (E-). Chickens in all three groups received ~10⁸
153 CFU *C. jejuni* by oral gavage at 18 days of age (C+). The differential dosing
154 schedule of nE+/C+ and aE+/C+ was to adjust for the different pre-patent periods of
155 these parasites, to ensure peak parasitaemia in the caeca at the time of *C. jejuni*
156 challenge in both groups. The non-attenuated and attenuated parasite lines were
157 used to compare the severity of pathology (i.e. presence/absence of the second
158 generation schizont) and the dose sizes were designed to reduce the confounding
159 effect of differential parasite replication, although it should be noted that equivalent

160 oocyst output was not expected (28). Parasite-associated pathology was only
161 anticipated for the non-attenuated Wis infected groups. Three days post *C. jejuni*
162 challenge (21 days of age) all birds were culled. Post-mortem caecal contents, liver,
163 and spleen tissue were collected immediately.

164 Trial 2 followed a similar experimental outline to trial 1 with Groups 1-3 receiving
165 identical treatment (nE+/C+, aE+/C+, and E-/C+ respectively, undertaken at RVC). In
166 addition, to directly compare the effect of *C. jejuni* challenge on parasite replication,
167 control groups received *E. tenella* treatment without *C. jejuni* challenge, using sterile
168 MH broth in place of *C. jejuni* (Groups 4-6; *E. tenella* Wis only: nE+/C-, *E. tenella*
169 WisF96 only: aE+/C-, no *E. tenella*: E-/C-). Groups 1-3 (all C+) comprised ten Light
170 Sussex chickens per group, while groups 4-6 (all C-) comprised six chickens per
171 group, reflecting the greater bird to bird variation in *C. jejuni* enumeration compared
172 to *E. tenella*. All birds were caged separately to facilitate collection of individual bird
173 faeces and enumeration of total daily oocyst output between 18 and 21 days of age
174 as described previously (32). All birds were culled three days post *C. jejuni* challenge
175 (21 days of age) and samples collected as described for trial 1.

176 Trial 3 was similar to trial 2, except that instead of using the attenuated *E. tenella*
177 WisF96 line, a low dose (400 oocysts) of non-attenuated *E. tenella* Wis was used to
178 assess the effect of parasite dose/replication, rather than reduced pathogenicity, on
179 the outcome of *C. jejuni* infection. In this trial, the culling of birds was delayed to ten
180 days post *C. jejuni* challenge to assess if the changes observed in *C. jejuni* load at
181 three days (Trials 1 and 2) were stable over a longer period. Additionally, to provide
182 a semi-quantitative comparison of bacterial load between trials 1, 2 and 3, birds were
183 swabbed cloacally three days post *C. jejuni* challenge, as described previously (11).
184 At 13 days of age, groups 1 and 4 received a high (h) dose of 4,000 sporulated *E.*

185 *tenella* Wis oocysts (nEh+/C+ and nEh+/C-) whilst groups 2 and 5 received a low (l)
186 dose of 400 sporulated *E. tenella* Wis oocysts (nEI+/C+ and nEI+/C-). Chickens in
187 groups 3 and 6 were not infected with the parasite (E-/C+ and E-/C-). At 18 days of
188 age groups 1, 3 and 5 were challenged with $\sim 10^8$ CFU *C. jejuni* whilst groups 2, 4
189 and 6 were mock challenged with sterile MH broth. Daily oocyst output was
190 assessed for each chicken between 18 and 22 days of age. Chickens were culled
191 ten days post bacterial challenge (28 days of age) and samples collected as
192 described for trial 1.

193 **Sample collection**

194 Post-mortem, 0.2-1.0 g of caecal contents, liver, and spleen were collected
195 aseptically from the same ~central part of each tissue/organ into universal tubes and
196 stored separately on ice prior to homogenisation in all trials. On the day of collection
197 all samples were weighed and homogenised in an equal volume (w/v) sterile PBS
198 using a TissueRuptor (Qiagen, Hilden, Germany), followed by serial 10-fold dilutions
199 in PBS. Additionally, ~3 cm tissue from the mid-point of one caeca, half the spleen,
200 and ~1 cm³ section of the mid-liver were recovered from chickens in trial 2 and
201 stored in RNAlater (Sigma) as recommended by the manufacturer for subsequent
202 RNA extraction and RT-qPCR.

203 **RNA extraction and integrity**

204 Total RNA was extracted from thawed tissue samples after storage at -20°C in
205 RNAlater using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the
206 manufacturer's instructions. The optional DNase digestion step was included to
207 remove contaminating genomic DNA. RNA concentration was determined using a
208 Nanodrop ND-2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA)

209 and samples were diluted in nuclease free water to produce a final concentration of
210 40 ng/ μ L. The quality of a sub-set of samples (~ 5%) was confirmed using an Agilent
211 RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) following the
212 manufacturer's instructions, confirming RNA Integrity Number results in excess of six
213 for further analysis.

214 **Real-time quantitative PCR (RT-qPCR)**

215 Superscript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, USA) was used to
216 make cDNA using total RNA purified from the samples collected, following the
217 manufacturer's instructions. Oligo (dT)₁₂₋₁₈ (Invitrogen, Carlsbad, USA) was used
218 along with the optional RNaseOut (Invitrogen) step. cDNA was used as template in
219 all RT-PCR reactions.

220 The oligonucleotide primer sequences used to target cDNA copies of each of the
221 mRNA transcripts investigated including mucin (MUC) 2, MUC 5ac, MUC 13, IL-1 β ,
222 IL-6, IFN γ , IL-2, IL-10, IL-13, inducible nitric-oxide (iNOS), and three reference
223 transcripts are summarised in Supplementary Table 1. The final reaction volumes for
224 RT-qPCR consisted of 10 μ l SsoFast EvaGreen super mix, containing Sybr Green
225 dye (Bio-Rad), 70 nM of each primer (Sigma-Aldrich), forward and reverse, and were
226 made up to 19 μ l using RNase and DNase free water (Invitrogen, Paisley, UK). To
227 one volume of this master-mix 1 μ l of cDNA was added. As a negative control, 1 μ l of
228 water was used in place of cDNA. DNA was amplified on a Bio-Rad CFX 2.0 cycler
229 (Bio-Rad) in triplicate, for every sample, using the following conditions; 1 cycle at
230 95 $^{\circ}$ C for 60 s followed by 40 cycles of 95 $^{\circ}$ C for 15 s and the appropriate annealing
231 temperature (as indicated in Supplementary Table 1) for 30 s. After completion, a
232 melt curve was generated by running one cycle at 65 $^{\circ}$ C for 0.05 s and 95 $^{\circ}$ C for 0.5

233 s. Individual transcripts were normalised individually to the three reference genes
234 and used to calculate a mean figure for each replicate. Briefly, quantification cycle
235 (Cq) values for each sample were generated using the BioRad CFX 2.0 software and
236 enabled quantification of cDNA when normalized to the reference genes,
237 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein
238 (TATA-BP), and 28S rRNA.

239 **Statistical analysis**

240 Statistical analyses including calculation of arithmetic means, associated standard
241 deviation or error of the mean, analysis of variance and associated post-hoc Tukeys
242 tests were performed using SPSS Statistics v24 (IBM). Bacterial counts were
243 logarithmically transformed. Differences were considered significant where $P < 0.05$.

244

245 **Results**

246 ***E. tenella*/*C. jejuni* co-infection**

247 For all three trials, at all sampling sites *C. jejuni* was not detected above the limit of
248 detection in any of the unchallenged (C-) birds.

249 Trial 1 (pilot, Table 1). In the caeca, three days post bacterial challenge, co-infection
250 with non-attenuated or attenuated *E. tenella* caused a significant 2.5 or 1 \log_{10}
251 increase in *C. jejuni* load ($P < 0.001$ and $P < 0.05$), respectively, compared to *C.*
252 *jejuni* alone. A significant difference in caecal *C. jejuni* colonisation was also
253 detected between the non-attenuated and attenuated parasite groups ($P < 0.05$). In
254 the spleen co-infection with either of the *E. tenella* lines caused a non-significant 1
255 \log_{10} decrease in *C. jejuni* load ($P > 0.05$) compared to *C. jejuni* alone. Similarly, in

256 the liver, co-infection with either parasite line caused a non-significant $\sim 1 \log_{10}$
257 decrease in *C. jejuni* load ($P > 0.05$).

258 Trial 2. In the caeca, three days post bacterial challenge, co-infection with non-
259 attenuated or attenuated *E. tenella* caused a significant 2.9 or 1.35 \log_{10} increase in
260 *C. jejuni* load, respectively, compared to *C. jejuni* alone ($P < 0.001$; Figure 1A). A
261 significant difference in *C. jejuni* colonisation was again detected in the caeca
262 between non-attenuated and attenuated parasite groups ($P < 0.001$). Here, *C. jejuni*
263 load was positively correlated with parasite replication, measured in terms of total
264 oocyst output ($r = 0.893$, $P < 0.001$; Figure 1E). In the liver co-infection with non-
265 attenuated and attenuated *E. tenella* caused a significant $\sim 1 \log_{10}$ decrease in *C.*
266 *jejuni* ($P < 0.05$; Figure 1B), although no difference was detected between the
267 parasite lines ($P > 0.05$). Similarly, in the spleen co-infection with either *E. tenella*
268 line caused a significant 1.8 or 1.1 \log_{10} decrease in *C. jejuni*, respectively, ($P < 0.05$;
269 Figure 1C), with no difference between parasite lines. In both liver and spleen no
270 association was detected between *C. jejuni* and the level of faecal oocyst output ($P >$
271 0.05 ; Figures 1F and G). Total oocyst output was higher in chickens infected with
272 non-attenuated *E. tenella* compared with the attenuated line (Figure 1D).

273 Trial 3 (Table 2). Cloacal swabs were collected three days post *C. jejuni* infection
274 from all groups. Co-infection initiated with a high non-attenuated *E. tenella* dose
275 caused a significant, 1.6 \log_{10} increase in cloacal *C. jejuni* ($P < 0.001$), compared to
276 *C. jejuni* alone. In the co-infected group with a low parasite dose, no difference in *C.*
277 *jejuni* load was observed ($P > 0.05$). A significant difference in cloacal *C. jejuni* load
278 was noted between the groups co-infected with high and low parasite doses ($P <$
279 0.001).

280 In the caeca, ten days post *C. jejuni* infection, co-infection initiated with a high *E.*
281 *tenella* dose caused a significant 1.5 log₁₀ increase in *C. jejuni* colonisation
282 compared to *C. jejuni* alone (P < 0.01). There was a significant association with
283 oocyst output (r = 0.682, P = 0.001). Co-infection with the low parasite dose group
284 did not cause a significant change in *C. jejuni* colonisation compared to *C. jejuni*
285 alone (P < 0.05). Significant variation in the level of *C. jejuni* colonisation was noted
286 between the high and low *E. tenella* groups (P < 0.01).

287 In the spleen, ten days post *C. jejuni* infection, no significant difference was detected
288 in the levels of *C. jejuni* between in the presence or absence of *E. tenella*, however a
289 non-significant (P > 0.05) decreasing trend in *C. jejuni* colonisation was observed.
290 No association was detected between *C. jejuni* in the spleen and the level of faecal
291 oocyst output (r = -0.44, P > 0.05).

292 In the liver, ten days post *C. jejuni* infection, there was a significant decrease in *C.*
293 *jejuni* colonisation in the high dose *E. tenella* group compared to *C. jejuni* alone (P <
294 0.05). No significant changes were observed from the low parasite dose. No
295 association was detected between *C. jejuni* in the liver and the level of faecal oocyst
296 output (r = -0.31, P > 0.05).

297 **Cytokine Response to *E. tenella*/*C. jejuni* Challenge**

298 *E. tenella* infection induces a strong immune response and it was postulated that the
299 changes in *C. jejuni* load noted in the co-infection models could be due to an
300 associated 'bystander' immune response. Caecal tissues collected during Trial 2 at
301 21 days of age were used to investigate the transcription of a variety of cytokines
302 (i.e. a single time point, equivalent to seven, five and three days after challenge by
303 Wis, WisF96 and *C. jejuni*, respectively). The transcriptional fold change of each

304 group compared to the uninfected control is summarised in Table 3, along with the
305 fold change of the co-infected groups, compared to the *C. jejuni* only group. Infection
306 with *C. jejuni* alone significantly increased transcription of IL-1 β and iNOS (both $P \leq$
307 0.001), as well as IL-13 ($P \leq 0.01$). Infection with non-attenuated or attenuated *E.*
308 *tenella* increased caecal transcription of IL-1 β , IL-2, IL-6, IL-10, iNOS and IFN γ
309 significantly when compared to uninfected and *C. jejuni* only infected groups,
310 irrespective of *C. jejuni* co-infection. Transcription of IL-13 was significantly
311 decreased in all *Eimeria* infected groups. Accompanying P values indicated in Table
312 3.

313 **Mucin Gene Transcription in Response to *E. tenella*/*C. jejuni* Challenge**

314 Caecal transcription of the mucin genes *muc2*, *muc5ac* and *muc13* was assessed to
315 explore the consequences of infection. *C. jejuni* infection alone resulted in no
316 difference in *muc* gene transcription three days post-challenge (Table 3). Infection
317 with non-attenuated *E. tenella* resulted in upregulation in *muc2*, *muc5ac* and *muc13*
318 transcription, most notably *muc5ac* which was the only *muc* gene significantly
319 upregulated during attenuated *E. tenella* infection.

320

321 **Discussion**

322 *In vivo* trials were carried out to analyse the impact of parasite co-infection on *C.*
323 *jejuni* colonisation of the caeca, spleen and liver of chickens. Local transcription of
324 selected cytokine and mucin genes was assessed in an effort to explain the
325 differences detected. It was hypothesised that damage to the caecal epithelial barrier
326 induced by the haemorrhagic parasite *E. tenella* and/or the consequential pro-
327 inflammatory immune response would facilitate increased bacterial colonisation in

328 the caeca, liver and spleen. Quantification of *C. jejuni* colonisation at these three
329 sites revealed significant variation in the presence or absence of concurrent *E.*
330 *tenella* infection, disproving the hypothesis for the liver and spleen. Parasite co-
331 infection was associated with elevated *C. jejuni* loads within the caecal contents, but
332 reduced loads in the liver and spleen. Thus, while faecal shedding of *C. jejuni* was
333 increased by concomitant *E. tenella* infection, deep tissue bacterial contamination
334 was decreased. This is in direct contrast to what has been observed when chickens
335 are co-infected with *Eimeria* parasites and either *C. perfringens* or *S. enterica*
336 Typhimurium (5, 10). It has been shown that *E. tenella* infection can influence the
337 caecal microflora in a manner that has been reported, by some (33, 34), to
338 potentially benefit *C. jejuni* colonisation and demonstrates that *E. tenella* induced
339 dysbiosis may increase susceptibility to enteric pathogens such as *C. jejuni*. Further
340 analysis of the microbiome of co-infected poultry is needed to investigate this
341 hypothesis. Increased bacterial load in the gut but not the internal organs due to co-
342 infection with globally enzootic *Eimeria* parasites (8) is relevant to the food safety risk
343 posed by *C. jejuni*. Furthermore, these results are pertinent to the development of
344 *Eimeria* as a novel vaccine vector system. This approach aims to utilise transgenic
345 attenuated strains of the parasite to deliver vaccine antigens to chickens. Live
346 attenuated vaccines are currently used to vaccinate over one billion birds each year
347 (11) and results from this study suggest that attenuated strains have the potential to
348 reduce *C. jejuni* colonisation in the liver of poultry, which could limit human cases of
349 campylobacteriosis. Paradoxically, increases in *C. jejuni* colonisation in the caeca
350 are of concern, although improvements in abattoir protocols have been associated
351 with a shift in the importance of surface contamination by faeces to deep tissue
352 colonisation by *C. jejuni*, exacerbated by the deliberate undercooking or sautéing of

353 chicken liver due to the belief this will enhance the flavour and appearance of the
354 end product (35).

355 It is well recognised that individually both *E. tenella* and *C. jejuni* generate an
356 immune response, of varying levels, in chickens following infection (24, 27, 36-38).
357 The impact of *E. tenella* co-infection on *C. jejuni* colonisation and concurrent effect
358 on cytokine production has not been reported. Previously wild type (non-attenuated)
359 strains of *E. tenella* have been shown to induce a significant immune response in
360 chickens (24, 27), which is far greater than that induced by *C. jejuni* alone (36, 37).
361 These findings were replicated in this study, where the transcription of all but one of
362 the cytokines tested, IL-13, were increased in nE/C- compared to E-/C+ chickens.
363 Additionally, in this study it is notable that there was a significant increase in the
364 transcription of the majority of cytokines investigated in aE/C- compared to E-/C+
365 chickens, despite considerable attenuation of the WisF96 parasite line. To the best
366 of our knowledge this is the first report of immune responses associated with *in vivo*
367 WisF96 infection. The induction of immune responses in the absence of significant
368 pathology is relevant to the efficacy of attenuated anticoccidial vaccines. It is
369 postulated that the reduction in *C. jejuni* colonisation in the liver and spleen in the co-
370 infection model could be due to an associated, 'bystander' immune response
371 induced by the parasite. *E. tenella* infection stimulates a strong pro-inflammatory
372 immune response including significant increases in IFN γ and iNOS (39). iNOS has
373 also been directly linked to the control of *C. jejuni* (40). Caecal iNOS transcription
374 was increased six- or eight-fold during infection with attenuated or non-attenuated *E.*
375 *tenella*. The up-regulation of immune factors linked to control of *C. jejuni* as a
376 consequence of an ongoing *E. tenella* infection may explain, at least in part, the
377 reduced translocation of *C. jejuni* to the liver and spleen in co-infected chickens.

378 IFN γ levels are balanced by anti-inflammatory cytokines such as IL-10 (41).
379 Humphrey et al. (2014) reported that regulation of IL-10 is important in controlling
380 intestinal pathology in *C. jejuni* infected chickens, where lower levels associated with
381 prolonged inflammation and diarrhoea (36). In support, Vaezrad et al. (2017)
382 demonstrated that using glucocorticoids to dampen the immune system of chickens
383 reduced expression of pro-inflammatory genes and increased the colonisation of *C.*
384 *jejuni* in the caeca as well as translocation to, and colonisation of the liver (42). The
385 work of Vaezrad et al. (2017) supports the hypothesis that the increase in *C. jejuni*
386 caecal colonisation may also be influenced by physical damage. *E. tenella* infection
387 causes sloughing of cells which form the epithelial barrier and this damage may
388 facilitate enhanced *C. jejuni* colonisation in the caeca, akin to the mechanism utilised
389 by *C. perfringens* to invade the gut in the presence of *Eimeria* (43, 44).

390 Increased transcription of the majority of cytokines in the caecal tissue in co-infected
391 birds did not appear to impede *C. jejuni* colonisation of the caecal contents, although
392 it is not clear if this was a cause or effect. These results suggest that the
393 mechanism(s) responsible for the increase in *C. jejuni* detected within the caecal
394 lumen is distinct from translocation through the caecal wall and/or deep tissue
395 colonisation. *E. tenella* can cause a haemorrhagic form of coccidiosis characterised
396 by large volumes of blood in the caeca (45). Iron is an essential nutrient for
397 colonisation of *C. jejuni*, however bioavailability is limited within many host
398 environments (46). Bacteria can take up iron via environmental sources, such as
399 haemin and haemoglobin (47). It is hypothesised that the increased availability of
400 haemoglobin in the caeca, due to epithelial damage caused by *E. tenella*, may have
401 provided *C. jejuni* with an increased source of iron facilitating enhanced growth and
402 replication. The apparent pathology-dependent effect between non-attenuated and

403 attenuated parasite infections supports such a hypothesis, and it is noted that the
404 attenuated line was expected to induce little or no haemorrhage. Attenuated *E.*
405 *tenella* are less pathogenic than the non-attenuated parasite (48) and cause less
406 damage to the intestinal epithelium, but still induce an equivalent immune response
407 (49). The subsequent comparison of high and low non-attenuated parasite doses
408 confirmed a dose-effect of *Eimeria* on *C. jejuni* colonisation within the caecal
409 contents, but not the liver or spleen, supporting the association between pathological
410 severity in the former but not the latter. While the parasite crowding effect is
411 expected to have reduced the scale of difference between the high and low doses by
412 the time of oocyst excretion (50), it is clear that pathology (lesion score) does
413 associate with dose level (51). Variation in unidentified immune factors may
414 contribute to this effect and could influence the increased caecal *C. jejuni* load in
415 chickens co-infected with the attenuated parasite, where caecal pathology would
416 have been minimal.

417 Trials one and two explored the impact of an ongoing infection with non-attenuated
418 or attenuated *E. tenella* on *C. jejuni* colonisation of chickens' three-days after
419 bacterial challenge. The healthy chicken caeca empties several times per day,
420 suggesting that the figures recorded represent true bacterial colonisation (52).
421 However, to confirm the association the study was repeated using a later sampling
422 point, revealing similar results at ten compared to three days post bacterial
423 challenge. Once *C. jejuni* contaminated food or faecal material is ingested by the
424 chicken transit time through the upper gastrointestinal tract is ~2.5 hours (53). Work
425 by Shaughnessy et al. (2009), using a similar inoculating dose to those used in this
426 study, showed high levels of persistent caecal colonisation at 6, 20 and 48 hours
427 post *C. jejuni* infection, indicating rapid colonisation of the bacteria in the caeca (38).

428 Meade et al. (2009) showed that the liver and spleen of the majority of birds were
429 colonised by *C. jejuni* 48 hours post infection (54). These studies support analysis of
430 *C. jejuni* colonisation in the *E. tenella* co-infection model three days post bacterial
431 infection, confirmed at ten days post infection. Practically, these results are also
432 relevant to the field situation where anticoccidial drugs are commonly withdrawn from
433 broiler diets three to five days prior to slaughter, indicating a risk of a parasite and
434 associated *C. jejuni* surge at the time of transportation and carcass processing.

435 In addition to haemorrhage, several *Eimeria* species have been associated with
436 enteric mucogenesis in chickens (5). *C. jejuni* has been shown to replicate rapidly in
437 intestinal mucus from chickens (21), suggesting that a mucogenic response may
438 encourage *Campylobacter* proliferation within the mucus layer. Bacterial proteins
439 required for motility and colonisation, including flagellin A and *Campylobacter*
440 invasion antigens, are known to be secreted in the presence of chicken mucus (55,
441 56). Chicken mucus has also been shown to enhance *C. jejuni* motility and
442 expression of the flagellar protein FlgR (57), to protect *C. jejuni* from some short and
443 medium-chain fatty acids (58) (59), and the viscous environment might aid binding
444 and invasion of mammalian cells (60). However, enteric mucus from chickens has
445 also been reported to attenuate *C. jejuni* 81-176 invasion of both avian and human
446 epithelial cells (61), possibly contributing to reduced translocation away from the
447 caeca. Mucins are a major component of mucus and in this study the transcription of
448 *muc2*, *muc5ac* (both secreted, mucus forming mucins (62)) and *muc13* (a
449 transmembrane mucin) increased in the presence of non-attenuated *E. tenella*.
450 Transcription of *muc5ac* was also increased during attenuated *E. tenella* infection. It
451 was therefore postulated that intestinal mucus could play a key role in the enteric
452 colonisation of *C. jejuni* in chickens and the interaction with *E. tenella*. A pilot study

453 investigating the impact of the mucus-thinning dietary supplement N-acetylcysteine
454 (NAC, Sigma-Aldrich) (63, 64) was carried out during an *in vivo* co-infection trial to
455 test this theory (summarised in Supplemental materials, Methods and
456 Supplementary Table 2). It was hypothesised that inclusion of a mucus-thinning
457 agent in the feed of chickens would balance *E. tenella* induced mucus secretion,
458 directly reducing nutrient availability in the caecal lumen and indirectly *C. jejuni*
459 replication and colonisation. Further, depleting the secreted mucus layer might be
460 expected to facilitate increased translocation to extra-intestinal sites such as the liver
461 and spleen. In mucin 2 deficient mice presenting with a diminished intestinal barrier,
462 infection and mortality caused by *S. enterica* serovar Typhimurium was increased
463 (65). Here, using periodic acid Schiff (PAS) staining it was not possible to detect any
464 consistent variation in the thickness or consistency of the intestinal mucus layer with
465 NAC supplementation. As a consequence no direct functional conclusions can be
466 drawn. However, NAC supplementation did abrogate the *E. tenella*-associated
467 increase in caecal *C. jejuni* load, with a further non-significant reduction in treated
468 compared to untreated single *C. jejuni* infected chickens. These results support the
469 view that chicken mucus may aid *C. jejuni* colonisation and/or replication, possibly
470 via the provision of nutrients required for sustained growth (66), but further work will
471 be required for confirmation. NAC supplementation is also likely to have exerted
472 other profound effects on the broader enteric microbiome, the influence of which is
473 not currently known. Interestingly, the significant decreases detected in *C. jejuni*
474 colonisation of the liver and spleen in the co-infection model were maintained in the
475 presence of NAC, suggesting either a limited role for mucus in this aspect of the
476 parasite-bacterial interaction or inefficacy of the NAC protocol.

477

478 **Conclusion**

479 The current study has demonstrated that *E. tenella* co-infection exerts a significant
480 impact on colonisation of *C. jejuni* in Light Sussex chickens, while upregulating
481 several relevant immune factors. Co-infection caused a significant increase in *C.*
482 *jejuni* colonisation in the caecal contents, in a parasite pathology and dose
483 dependent manner, but a decrease in the liver and spleen. Results were
484 reproducible on days three and ten post-bacterial challenge, highlighting the stability
485 of the effect. Investigation into the levels of mucin transcription suggested that the
486 presence of a depleted intestinal mucosal barrier may contribute. Similar co-infection
487 studies with broiler chickens raised under intensive conditions are required to assess
488 if these results are reproducible in a commercial setting. Building on these studies,
489 the influence of eimerian infection on *C. jejuni* colonisation of poultry may impact
490 both the use of live anticoccidial vaccines and the development of *Eimeria* as a novel
491 vaccine vector.

492

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504

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691

692 **Figure legends**

693 **Figure 1.** (A-C) *C. jejuni* load in single or co-infected Light Sussex chickens (Trial 2).

694 Circle = count per bird (\log_{10}). X = average count per treatment group (\log_{10}). (A)

695 Caecal contents. (B) Liver. (C) Spleen. (D) Total \log_{10} *E. tenella* oocyst output per

696 bird (circle) and average per group (X). (E-G) Relationship between *C. jejuni* load

697 and *E. tenella* oocyst output. Solid markers = non-attenuated *E. tenella*, hollow

698 markers = attenuated *E. tenella*. (E) Caecal contents. (F) Liver. (G) Spleen. (Key)

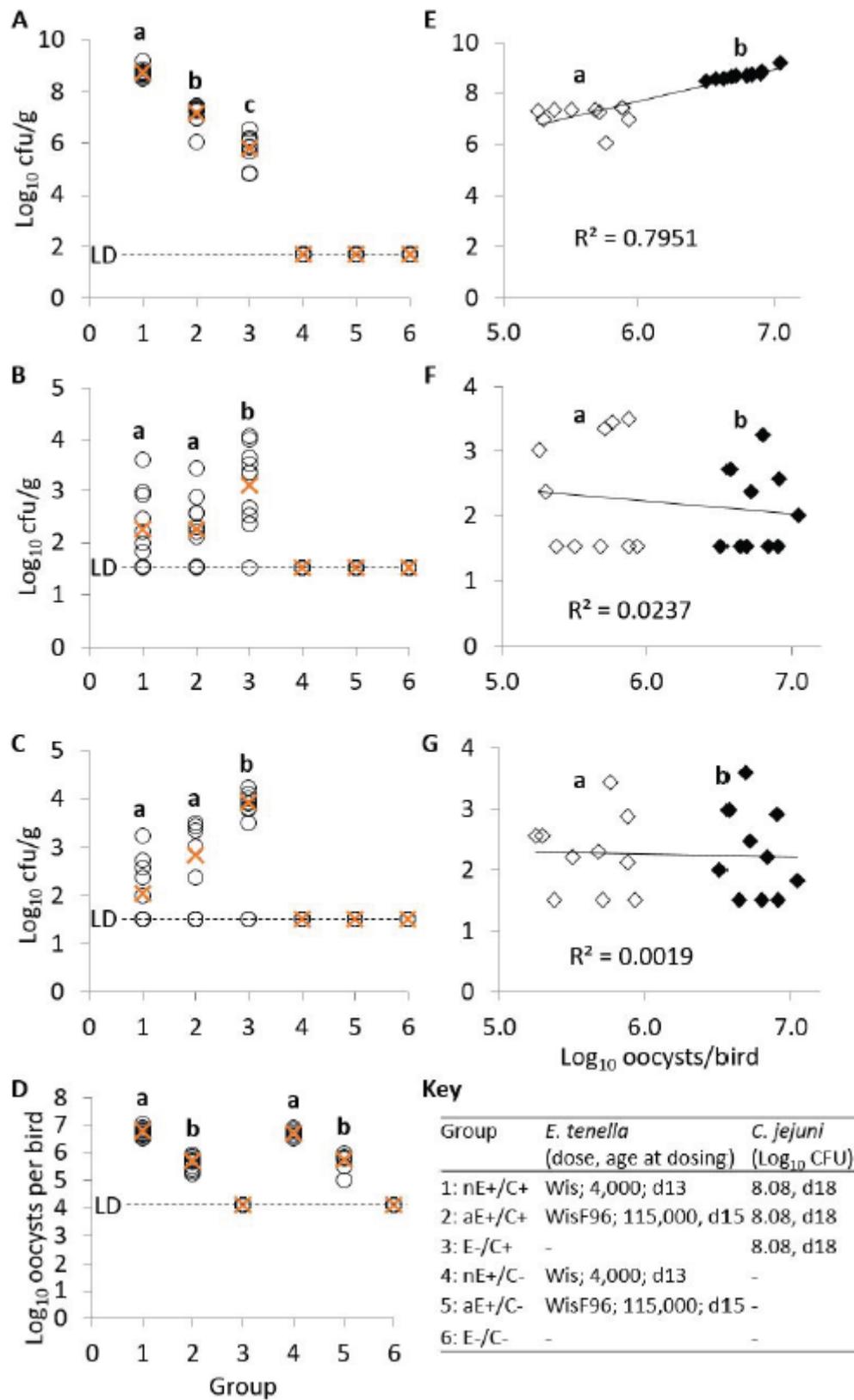
699 Group identifiers and experimental schedule. nE = non-attenuated *E. tenella*

700 Wisconsin, aE = attenuated *E. tenella* WisF96, C = *C. jejuni* 81-176. + =

701 administered. - = not administered, mock control. LD = limit of detection. Groups with

702 different superscript letters within plot indicate significant statistical differences.

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705

706 **Table 1.** *Campylobacter jejuni* and *Eimeria tenella* dose regimes and viable counts from single and co-infection of chickens in Trial
 707 1.

Group ¹	<i>E. tenella</i> strain (dose; age at dosing)	<i>C. jejuni</i> Log ₁₀ CFU (d18)	Log ₁₀ CFU/g Day 21 (three days post <i>C. jejuni</i>) Average ± SD		
			Caeca	Liver	Spleen
nE+/C+	Wis (4,000; d13)	8.17	9.13 ± 0.19 ^a	2.03 ± 1.22 ^a	1.67 ± 1.51 ^a
aE+/C+	WisF96 (115,000; d15)	8.17	7.55 ± 0.62 ^b	2.03 ± 1.23 ^a	1.35 ± 1.20 ^a
E-/C+	None	8.17	6.61 ± 1.77 ^c	2.91 ± 1.53 ^a	2.70 ± 1.71 ^a

708 nE = non-attenuated *E. tenella* Wis, aE = attenuated *E. tenella* WisF96, C = *C. jejuni*, + = administered, - = not administered. ¹= 8
 709 birds/group. Averages that were significantly different within each column are identified by a different superscript letter (p < 0.05).

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 711

712 **Table 2.** *Campylobacter jejuni* and *Eimeria tenella* dose regimes and viable counts from single and co-infection of chickens in Trial

713 3.

Group ¹	<i>E. tenella</i> strain (dose; age at dosing)	<i>C. jejuni</i> Log ₁₀ CFU (d18)	Log ₁₀ output oocysts per bird	Log ₁₀ CFU/g (Average ± SD)			
				Day 21 ²		Day 28 ³	
				Cloacal swab	Caeca	Liver	Spleen
nEh+/C+	Wis (4000; d13)	8.27	7.28 ± 0.06 ^a	9.16 ± 0.51 ^a	8.47 ± 0.51 ^a	1.99 ± 0.19 ^a	2.42 ± 0.50 ^a
nEI+/C+	Wis (400; d13)	8.27	6.75 ± 0.09 ^b	7.64 ± 0.49 ^b	7.05 ± 0.93 ^b	2.72 ± 0.26 ^{ab}	2.60 ± 0.47 ^a
E-/C+	None	8.27	nd	7.56 ± 0.54 ^b	6.97 ± 1.03 ^b	3.06 ± 0.32 ^b	3.27 ± 0.82 ^a
nEh+/C-	Wis (4000; d13)	Mock	7.28 ± 0.04 ^a	nd	nd	nd	nd
nEI+/C-	Wis (400; d13)	Mock	6.73 ± 0.07 ^b	nd	nd	nd	nd
E-/C-	None	Mock	nd	nd	nd	nd	nd

714 nE = non-attenuated *E. tenella* Wis, C = *C. jejuni*, h = high dose, l = low dose, + = administered, - = not administered, nd = none
715 detected. ¹= 8 birds/group. ²Sampled three days post-*C. jejuni* inoculation. ³Sampled ten days post-*C. jejuni* inoculation. Averages
716 that were significantly different within each column are identified by a different superscript letter (p < 0.05). Mock = no bacterial
717 control.

718

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720

721 **Table 3.** Transcriptional fold change of cytokines and mucins in caecal tissue collected during Trial 2.

Target Gene	Fold change (\pm SEM) versus uninfected					Fold change (\pm SEM) versus <i>C. jejuni</i> only infected group	
	nE+/C+	aE+/C+	E-/C+	nE+/C-	aE+/C-	nE+/C+	aE+/C+
IL-1 β	11.88 ^{***} \pm 0.55	11.33 ^{**} \pm 0.71	8.4 ^{***} \pm 0.40	10.6 ^{***} \pm 0.62	11.1 ^{***} \pm 0.97	1.42 ^{***} \pm 0.06	1.35 ^{**} \pm 0.08
IL-2	11.87 ^{***} \pm 0.88	10.37 ^{***} \pm 1.01	3.07 ^{ns} \pm 0.17	10.03 ^{***} \pm 0.73	7.97 ^{***} \pm 0.79	3.87 ^{***} \pm 0.29	3.38 ^{***} \pm 0.33
IL-6	18.86 ^{***} \pm 1.36	20.24 ^{***} \pm 1.15	3.83 ^{ns} \pm 0.20	18.12 ^{***} \pm 1.66	14.37 ^{***} \pm 1.27	4.92 ^{***} \pm 0.35	5.28 ^{***} \pm 0.30
IL-10	9.89 ^{***} \pm 0.78	9.06 ^{***} \pm 0.61	2.09 ^{ns} \pm 0.15	8.18 ^{***} \pm 1.13	8.97 ^{***} \pm 0.91	4.74 ^{***} \pm 0.37	4.34 ^{***} \pm 0.29
IL-13	-20 ^{***} \pm 0.003	-16.67 ^{***} \pm 0.004	1.34 ^{**} \pm 0.09	-25 ^{***} \pm 0.004	-16.67 ^{***} \pm 0.006	-27.03 ^{***} \pm 0.003	-21.01 ^{***} \pm 0.003
iNOS	8.72 ^{***} \pm 0.43	6.33 ^{***} \pm 0.31	4.56 ^{***} \pm 0.26	8.73 ^{***} \pm 0.60	5.94 ^{***} \pm 0.32	1.91 ^{***} \pm 0.09	1.39 ^{**} \pm 0.06
IFN γ	34.60 ^{***} \pm 1.84	29.96 ^{***} \pm 1.42	5.02 ^{ns} \pm 0.18	35.37 ^{***} \pm 1.54	32.84 ^{***} \pm 1.16	6.89 ^{***} \pm 0.37	5.96 ^{***} \pm 0.28
MUC2	1.41 ^{***} \pm 0.06	1.19 ^{ns} \pm 0.05	1.00 ^{ns} \pm 0.04	1.41 ^{**} \pm 0.06	1.16 ^{ns} \pm 0.04	1.41 ^{***} \pm 0.06	1.19 ^{ns} \pm 0.06
MUC5ac	3.27 ^{***} \pm 0.23	2.75 ^{***} \pm 0.15	1.22 ^{ns} \pm 0.10	3.16 ^{***} \pm 0.19	2.69 ^{***} \pm 0.15	2.68 ^{***} \pm 0.18	2.25 ^{***} \pm 0.12
MUC13	1.83 ^{***} \pm 0.11	1.33 ^{ns} \pm 0.09	1.20 ^{ns} \pm 0.08	1.82 ^{***} \pm 0.06	1.34 ^{ns} \pm 0.07	1.53 ^{***} \pm 0.10	1.11 ^{ns} \pm 0.08

722 nE = non-attenuated *E. tenella* Wis, aE = attenuated *E. tenella* WisF96, C = *C. jejuni*, + = administered, - = not administered. Fold change data that were
723 significantly different are identified by asterisks (ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Samples were collected 3 days post *C. jejuni* challenge.