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

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

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Abstract

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

Introduction

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

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

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

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

Parasites and propagation

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

Bacterial propagation

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

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

Experimental design

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

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

In trial 1 (pilot study, conducted at the Institute for Animal Health), 24 SPF Light Sussex chickens were caged in three groups of eight. Chickens in Group 1 received 4,000 sporulated *E. tenella* Wis (non-attenuated, n) oocysts by oral gavage at 13 days of age (nE+). Chickens in Group 2 received 115,000 sporulated WisF96 (attenuated; a) oocysts by oral gavage at 15 days of age (aE+). Chickens in Group 3 were not infected with *E. tenella* (E-). Chickens in all three groups received ~10^8 CFU *C. jejuni* by oral gavage at 18 days of age (C+). The differential dosing schedule of nE+/C+ and aE+/C+ was to adjust for the different pre-patent periods of these parasites, to ensure peak parasitaemia in the caeca at the time of *C. jejuni* challenge in both groups. The non-attenuated and attenuated parasite lines were used to compare the severity of pathology (i.e. presence/absence of the second generation schizont) and the dose sizes were designed to reduce the confounding 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 (nEl+/C+ and nEl+/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 ~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 cm^3 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)

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

Real-time quantitative PCR (RT-qPCR)

Superscript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, USA) was used to make cDNA using total RNA purified from the samples collected, following the manufacturer's instructions. Oligo $(dT)_{12\text{-}18}$ (Invitrogen, Carlsbad, USA) was used along with the optional RNaseOut (Invitrogen) step. cDNA was used as template in all RT-PCR reactions.

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

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

Statistical analysis

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

Results

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

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

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

In the caeca, ten days post *C. jejuni* infection, co-infection initiated with a high *E. tenella* dose caused a significant 1.5 $\log_{10}$ increase in *C. jejuni* colonisation compared to *C. jejuni* alone ($P < 0.01$). There was a significant association with oocyst output ($r = 0.682$, $P = 0.001$). Co-infection with the low parasite dose group did not cause a significant change in *C. jejuni* colonisation compared to *C. jejuni* alone ($P < 0.05$). Significant variation in the level of *C. jejuni* colonisation was noted between the high and low *E. tenella* groups ($P < 0.01$).

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

Conclusion

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

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References

1. Clark EL, Tomley FM, Blake DP. 2017. Are Eimeria Genetically Diverse, and Does It Matter? Trends Parasitol 33:231-241.
2. FAOstat. 2018. Food and Agriculture Organization of the United Nations FAOstat database. http://faostat3.fao.org/home/E. Accessed 15th October 2015.
3. Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen HL, Haring VR, Moore RJ. 2012. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Applied Microbiology and Biotechnology 96:1361-1369.
4. Rubio LA, Peinado MJ, Ruiz R, Suarez-Pereira E, Mellet CO, Fernandez JMG. 2015. Correlations between changes in intestinal microbiota composition and performance parameters in broiler chickens. Journal of Animal Physiology and Animal Nutrition 99:418-423.
5. Collier CT, Hofacre CL, Payne AM, Anderson DB, Kaiser P, Mackie RI, Gaskins HR. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet Immunol Immunopathol 122:104-15.
6. Awad WA, Dublecz F, Hess C, Dublecz K, Khayal B, Aschenbach JR, Hess M. 2016. Campylobacter jejuni colonization promotes the translocation of Escherichia coli to extra-intestinal organs and disturbs the short-chain fatty acids profiles in the chicken gut. Poult Sci doi:10.3382/ps/pew151.
7. Bull SA, Thomas A, Humphrey T, Ellis-Iversen J, Cook AJ, Lovell R, Jorgensen F. 2008. Flock health indicators and Campylobacter spp. in commercial housed broilers reared in Great Britain. Appl Environ Microbiol 74:5408-13.
8. Clark EL, Macdonald SE, Thenmozhi V, Kundu K, Garg R, Kumar S, Ayoade S, Fornace KM, Jatau ID, Moftah A, Nolan MJ, Sudhakar NR, Adebambo AO, Lawal IA, Alvarez Zapata R, Awuni JA, Chapman HD, Karimuribo E, Mugasa CM, Namangala B, Rushton J, Suo X, Thangaraj K, Srinivasa Rao AS, Tewari AK, Banerjee PS, Dhinakar Raj G, Raman M, Tomley FM, Blake DP. 2016. Cryptic Eimeria genotypes are common across the southern but not northern hemisphere. Int J Parasitol 46:537-44.
9. Blake DP, Tomley FM. 2014. Securing poultry production from the ever-present Eimeria challenge. Trends in Parasitology 30:12-19.
10. Baba E, Fukata T, Arakawa A. 1982. Establishment and persistence of *Salmonella* typhimurium infection stimulated by *Eimeria tenella* in chickens. Res Vet Sci 33:95-8.
11. Clark JD, Oakes RD, Redhead K, Crouch CF, Francis MJ, Tomley FM, Blake DP. 2012. Eimeria species parasites as novel vaccine delivery vectors: anti-Campylobacter jejuni protective immunity induced by Eimeria tenella-delivered CjaA. Vaccine 30:2683-8.
12. Marugan-Hernandez V, Cockle C, Macdonald S, Pegg E, Crouch C, Blake DP, Tomley FM. 2016. Viral proteins expressed in the protozoan parasite *Eimeria tenella* are detected by the chicken immune system. Parasit Vectors 9:463.
13. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B, World Health Organization Foodborne Disease Burden Epidemiology Reference G. 2015. World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. PLoS Med 12:e1001923.

14. Nielsen LN, Sheppard SK, McCarthy ND, Maiden MC, Ingmer H, Krogfelt KA. 2010. MLST clustering of Campylobacter jejuni isolates from patients with gastroenteritis, reactive arthritis and Guillain-Barre syndrome. J Appl Microbiol 108:591-9.
15. Skarp CPA, Hanninen ML, Rautelini HIK. 2016. Campylobacteriosis: the role of poultry meat. Clinical Microbiology and Infection 22:103-109.
16. Newell DG, Fearnley C. 2003. Sources of Campylobacter colonization in broiler chickens. Appl Environ Microbiol 69:4343-51.
17. Johnsen G, Kruse H, Hofshagen M. 2006. Genetic diversity and description of transmission routes for Campylobacter on broiler farms by amplified-fragment length polymorphism. J Appl Microbiol 101:1130-9.
18. Cawthraw SA, Newell DG. 2010. Investigation of the Presence and Protective Effects of Maternal Antibodies Against Campylobacter jejuni in Chickens. Avian Diseases 54:86-93.
19. Young KT, Davis LM, Dirita VJ. 2007. Campylobacter jejuni: molecular biology and pathogenesis. Nat Rev Microbiol 5:665-79.
20. Hermans D, Van Deun K, Martel A, Van Immerseel F, Messens W, Heyndrickx M, Haesebrouck F, Pasmans F. 2011. Colonization factors of Campylobacter jejuni in the chicken gut. Veterinary Research 42.
21. Van Deun K, Pasmans F, Ducatelle R, Flahou B, Vissenberg K, Martel A, Van den Broeck W, Van Immerseel F, Haesebrouck F. 2008. Colonization strategy of Campylobacter jejuni results in persistent infection of the chicken gut. Veterinary Microbiology 130:285-97.
22. Hutchison M, Harrison D, Richardson I, Tchorzewska M. 2015. A Method for the Preparation of Chicken Liver Pate that Reliably Destroys Campylobacters. Int J Environ Res Public Health 12:4652-69.
23. Little CL, Gormley FJ, Rawal N, Richardson JF. 2010. A recipe for disaster: outbreaks of campylobacteriosis associated with poultry liver pate in England and Wales. Epidemiol Infect 138:1691-4.
24. Hong YH, Lillehoj HS, Lee SH, Dalloul RA, Lillehoj EP. 2006. Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Vet Immunol Immunopathol 114:209-23.
25. Rose ME, Hesketh P, Wakelin D. 1992. Immune control of murine coccidiosis: CD4+ and CD8+ T lymphocytes contribute differentially in resistance to primary and secondary infections. Parasitology 105 (Pt 3):349-54.
26. Vervelde L, Vermeulen AN, Jeurissen SH. 1996. In situ characterization of leucocyte subpopulations after infection with Eimeria tenella in chickens. Parasite Immunol 18:247-56.
27. Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, Smith AL, Kaiser P. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J Immunol 173:2675-2682.
28. McDougald LR, Jeffers TK. 1976. Comparative in vitro development of precocious and normal strains of Eimeria tenella (Coccidia). J Protozool 23:530-4.
29. McDougald LR, Jeffers TK. 1976. *Eimeria tenella* (Sporozoa, Coccidia): Gametogony following a single asexual generation. Science 192:258-9.
30. Long P, Joyner L, Millard B, Norton C. 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Veterinaria Latina 6:201-217.
31. Guccione E, Leon-Kempis Mdel R, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, Stevens MP, Kelly DJ. 2008. Amino acid-dependent growth of Campylobacter jejuni: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. Mol Microbiol 69:77-93.
32. Blake DP, Hesketh P, Archer A, Shirley MW, Smith AL. 2006. *Eimeria maxima*: the influence of host genotype on parasite reproduction as revealed by quantitative real-time PCR. Int J Parasitol 36:97-105.

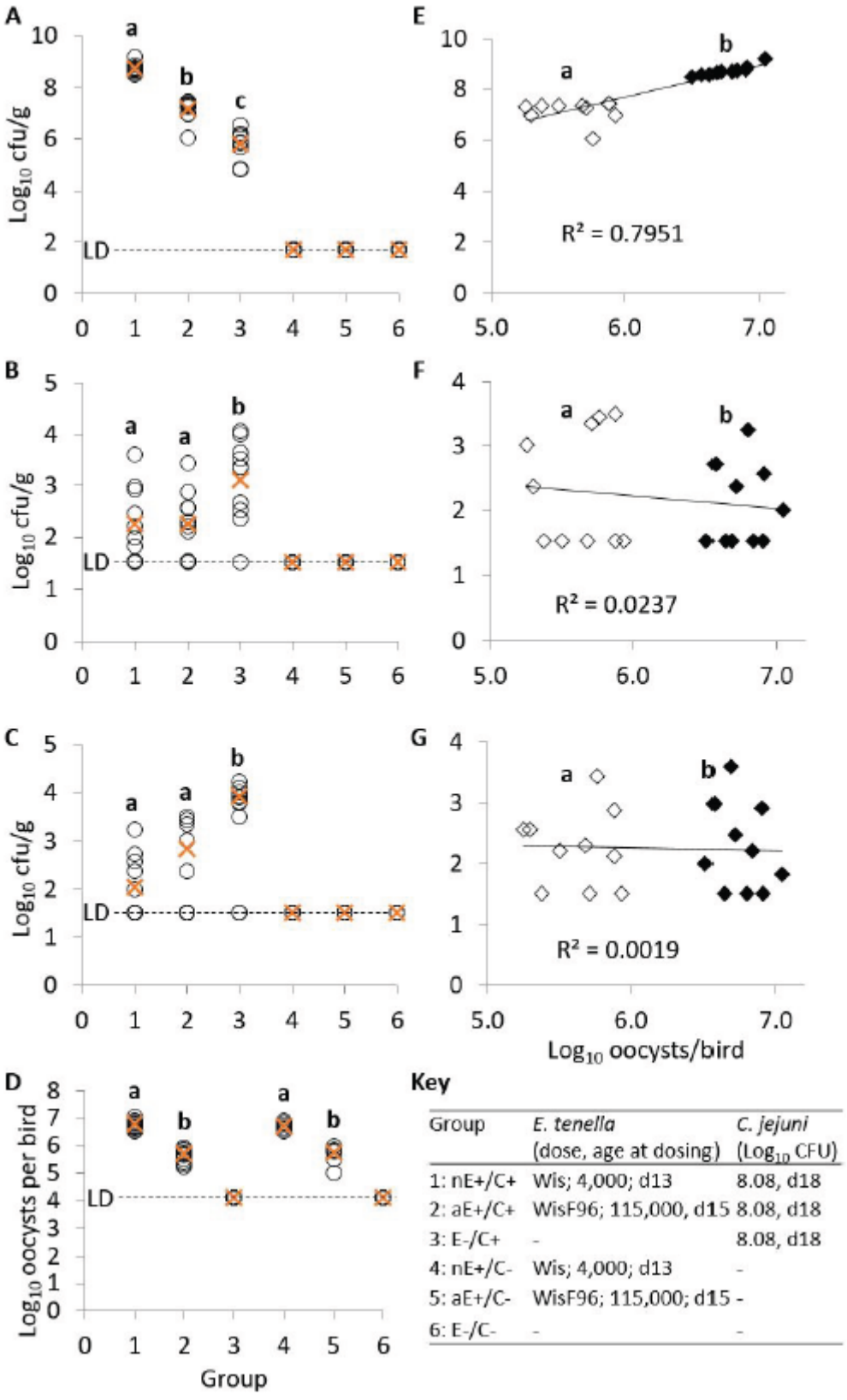
33. Bereswill S, Plickert R, Fischer A, Kuhl AA, Loddenkemper C, Batra A, Siegmund B, Gobel UB, Heimesaat MM. 2011. What you eat is what you get: Novel Campylobacter models in the quadrangle relationship between nutrition, obesity, microbiota and susceptibility to infection. Eur J Microbiol Immunol (Bp) 1:237-48.
34. Macdonald SE, Nolan MJ, Harman K, Boulton K, Hume DA, Tomley FM, Stabler RA, Blake DP. 2017. Effects of *Eimeria tenella* infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. PLoS One 12:e0184890.
35. Jones AK, Rigby D, Burton M, Millman C, Williams NJ, Jones TR, Wigley P, O'Brien SJ, Cross P, Consortium E. 2016. Restaurant Cooking Trends and Increased Risk for Campylobacter Infection. Emerg Infect Dis 22:1208-15.
36. Humphrey S, Chaloner G, Kemmett K, Davidson N, Williams N, Kipar A, Humphrey T, Wigley P. 2014. Campylobacter jejuni is not merely a commensal in commercial broiler chickens and affects bird welfare. MBio 5:e01364-14.
37. Smith CK, Abuoun M, Cawthraw SA, Humphrey TJ, Rothwell L, Kaiser P, Barrow PA, Jones MA. 2008. Campylobacter colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunol Med Microbiol 54:114-21.
38. Shaughnessy RG, Meade KG, Cahalane S, Allan B, Reiman C, Callanan JJ, O'Farrelly C. 2009. Innate immune gene expression differentiates the early avian intestinal response between Salmonella and Campylobacter. Vet Immunol Immunopathol 132:191-8.
39. Laurent F, Mancassola R, Lacroix S, Menezes R, Naciri M. 2001. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect Immun 69:2527-2534.
40. Iovine NM, Pursnani S, Voldman A, Wasserman G, Blaser MJ, Weinrauch Y. 2008. Reactive nitrogen species contribute to innate host defense against Campylobacter jejuni. Infect Immun 76:986-93.
41. Wu Z, Hu T, Rothwell L, Vervelde L, Kaiser P, Boulton K, Nolan MJ, Tomley FM, Blake DP, Hume DA. 2016. Analysis of the function of IL-10 in chickens using specific neutralising antibodies and a sensitive capture ELISA. Dev Comp Immunol 63:206-12.
42. Vaezirad MM, Keestra-Gounder AM, de Zoete MR, Koene MG, Wagenaar JA, van Putten JPM. 2017. Invasive behavior of Campylobacter jejuni in immunosuppressed chicken. Virulence 8:248-260.
43. Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol 40:341-7.
44. Witlock DR, Lushbaugh WB, Danforth HD, Ruff MD. 1975. Scanning electron microscopy of the cecal mucosa in Eimeria-tenella-infected and uninfected chickens. Avian Dis 19:293-304.
45. Witlock DR. 1983. Physiologic basis of blood loss during Eimeria tenella infection. Avian Dis 27:1043-50.
46. Palyada K, Threadgill D, Stintzi A. 2004. Iron acquisition and regulation in Campylobacter jejuni. Journal of Bacteriology 186:4714-4729.
47. Pickett CL, Auffenberg T, Pesci EC, Sheen VL, Jusuf SSD. 1992. Iron Acquisition and Hemolysin Production by Campylobacter-Jejuni. Infection and Immunity 60:3872-3877.
48. Shirley MW, Millard BJ. 1986. Studies on the Immunogenicity of 7 Attenuated Lines of Eimeria Given as a Mixture to Chickens. Avian Pathology 15:629-638.
49. McDonald V, Shirley MW, Millard BJ. 1986. A comparative study of two lines of Eimeria tenella attenuated either by selection for precocious development in the chicken or by growth in chicken embryos. Avian Pathol 15:323-35.
50. Williams RB. 2001. Quantification of the crowding effect during infections with the seven *Eimeria* species of the domesticated fowl: its importance for experimental designs and the production of oocyst stocks. Int J Parasitol 31:1056-69.

51. Nolan MJ, Tomley FM, Kaiser P, Blake DP. 2015. Quantitative real-time PCR (qPCR) for *Eimeria tenella* replication - Implications for experimental refinement and animal welfare. Parasitol Int 64:464-470.
52. Stanley D, Geier MS, Chen H, Hughes RJ, Moore RJ. 2015. Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. BMC Microbiol 15:51.
53. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. 2014. Extensive Microbial and Functional Diversity within the Chicken Cecal Microbiome. Plos One 9.
54. Meade K, Narciandi F, Cahalane S, Reiman C, Allan B, O'Farrelly C. 2009. Comparative in vivo infection models yield insights on early host immune response to Campylobacter in chickens. Immunogenetics 61:101-110.
55. Biswas D, Fernando UM, Reiman CD, Willson PJ, Townsend HGG, Potter AA, Allan BJ. 2007. Correlation between in vitro secretion of virulence-associated proteins of Campylobacter jejuni and colonization of chickens. Curr Microbiol 54:207-212.
56. Wassenaar TM, Vanderzeijst BAM, Ayling R, Newell DG. 1993. Colonization of Chicks by Motility Mutants of Campylobacter-Jejuni Demonstrates the Importance of Flagellin-a Expression. Journal of General Microbiology 139:1171-1175.
57. Shortt C, Scanlan E, Hilliard A, Cotroneo CE, Bourke B, Croinin TO. 2016. DNA Supercoiling Regulates the Motility of Campylobacter jejuni and Is Altered by Growth in the Presence of Chicken Mucus. Mbio 7.
58. Hermans D, Martel A, Van Deun K, Verlinden M, Van Immerseel F, Garmyn A, Messens W, Heyndrickx M, Haesebrouck F, Pasmans F. 2010. Intestinal mucus protects Campylobacter jejuni in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids. Poult Sci 89:1144-55.
59. Van Deun K, Haesebrouck F, Van Immerseel F, Ducatelle R, Pasmans F. 2008. Short-chain fatty acids and L-lactate as feed additives to control Campylobacter jejuni infections in broilers. Avian Pathology 37:379-383.
60. Szymanski CM, King M, Haardt M, Armstrong GD. 1995. Campylobacter-Jejuni Motility and Invasion of Caco-2 Cells. Infection and Immunity 63:4295-4300.
61. Byrne CM, Clyne M, Bourke B. 2007. Campylobacter jejuni adhere to and invade chicken intestinal epithelial cells in vitro. Microbiology 153:561-9.
62. Lang T, Hansson GC, Samuelsson T. 2006. An inventory of mucin genes in the chicken genome shows that the mucin domain of Muc13 is encoded by multiple exons and that ovomucin is part of a locus of related gel-forming mucins. BMC Genomics 7:197.
63. Hyman HC, Levisohn S, Yogev D, Razin S. 1989. DNA probes for Mycoplasma gallisepticum and Mycoplasma synoviae: application in experimentally infected chickens. Vet Microbiol 20:323-37.
64. De Lisle RC, Roach E, Jansson K. 2007. Effects of laxative and N-acetylcysteine on mucus accumulation, bacterial load, transit, and inflammation in the cystic fibrosis mouse small intestine. Am J Physiol Gastrointest Liver Physiol 293:G577-84.
65. Zarepour M, Bhullar K, Montero M, Ma C, Huang T, Velcich A, Xia L, Vallance BA. 2013. The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella enterica serovar Typhimurium colitis. Infect Immun 81:3672-83.
66. Stahl M, Vallance BA. 2015. Insights into Campylobacter jejuni colonization of the mammalian intestinal tract using a novel mouse model of infection. Gut Microbes 6:143-8.

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.

703


A
B
C
D
E
F
G
Log10 cfu/g
Log10 oocysts per bird
Log10 oocysts/bird
Group
LD
a
b
c
R² = 0.7951
R² = 0.0237
R² = 0.0019
Key
Group | E. tenella (dose, age at dosing) | C. jejuni (Log10 CFU)
1: nE+/C+ | Wis; 4,000; d13 | 8.08, d18
2: aE+/C+ | WisF96; 115,000, d15 | 8.08, d18
3: E-/C+ | - | 8.08, d18
4: nE+/C- | Wis; 4,000; d13 | -
5: aE+/C- | WisF96; 115,000; d15 | -
6: E-/C- | - | -

704

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]	*E. tenella* strain (dose; age at dosing)	*C. jejuni* Log_{10} CFU (d18)	Log_{10} CFU/g Day 21 (three days post *C. jejuni)* 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 ($p < 0.05$).

710
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]	*E. tenella* strain (dose; age at dosing)	*C. jejuni* 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}
nEl+/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
nEl+/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. [2]Sampled three days post-*C. jejuni* inoculation. [3]Sampled 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
719
720

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

	Fold change (±SEM) versus uninfected					Fold change (±SEM) versus *C. jejuni* only infected group	
Target Gene	nE+/C+	aE+/C+	E-/C+	nE+/C-	aE+/C-	nE+/C+	aE+/C+
IL-1β	11.88*** ±*0.55*	11.33**±*0.71*	8.4***±*0.40*	10.6***±*0.62*	11.1*** ±*0.97*	1.42***±*0.06*	1.35**±*0.08*
IL-2	11.87***±*0.88*	10.37***±*1.01*	3.07 ^{ns}±*0.17*	10.03***±*0.73*	7.97***±*0.79*	3.87***±*0.29*	3.38***±*0.33*
IL-6	18.86***±*1.36*	20.24***±*1.15*	3.83^{ns}±*0.20*	18.12***±*1.66*	14.37***±*1.27*	4.92***±*0.35*	5.28***±*0.30*
IL-10	9.89***±*0.78*	9.06***±*0.61*	2.09 ^{ns}±*0.15*	8.18***±*1.13*	8.97***±*0.91*	4.74***±*0.37*	4.34***±*0.29*
IL-13	-20***±*0.003*	-16.67***±*0.004*	1.34 **±*0.09*	-25***±*0.004*	-16.67***±*0.006*	-27.03***±*0.003*	-21.01***±*0.003*
iNOS	8.72***±*0.43*	6.33***±*0.31*	4.56***±*0.26*	8.73***±*0.60*	5.94***±*0.32*	1.91***±*0.09*	1.39**±*0.06*
IFNγ	34.60***±*1.84*	29.96***±*1.42*	5.02^{ns}±*0.18*	35.37***±*1.54*	32.84***±*1.16*	6.89***±*0.37*	5.96***±*0.28*
MUC2	1.41***±*0.06*	1.19^{ns}±*0.05*	1.00^{ns}±*0.04*	1.41**±*0.06*	1.16^{ns}±*0.04*	1.41***±*0.06*	1.19^{ns}±*0.06*
MUC5ac	3.27***±*0.23*	2.75***±*0.15*	1.22^{ns}±*0.10*	3.16***±*0.19*	2.69***±*0.15*	2.68***±*0.18*	2.25***±*0.12*
MUC13	1.83***±*0.11*	1.33^{ns}±*0.09*	1.20^{ns}±*0.08*	1.82***±*0.06*	1.34^{ns}±*0.07*	1.53***±*0.10*	1.11^{ns}±*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 ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001). Samples were collected 3 days post *C. jejuni* challenge.