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).

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

Materials and Methods

Ethics statement

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

Animals

Light Sussex chickens, purchased from the Institute for Animal Health Poultry Production Unit (IAH PPU, Compton, UK) were used for all experiments. All chickens 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

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

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

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

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

Sample collection

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

RNA extraction and integrity

Total RNA was extracted from thawed tissue samples after storage at -20°C in RNAlater using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The optional DNase digestion step was included to remove contaminating genomic DNA. RNA concentration was determined using a 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. tenellaC. 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

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

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

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

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

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

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

It is well recognised that individually both *E. tenella* and *C. jejuni* generate an immune response, of varying levels, in chickens following infection (24, 27, 36-38). The impact of *E. tenella* co-infection on *C. jejuni* colonisation and concurrent effect on cytokine production has not been reported. Previously wild type (non-attenuated) strains of *E. tenella* have been shown to induce a significant immune response in chickens (24, 27), which is far greater than that induced by *C. jejuni* alone (36, 37). These findings were replicated in this study, where the transcription of all but one of the cytokines tested, IL-13, were increased in nE/C- compared to E-/C+ chickens. Additionally, in this study it is notable that there was a significant increase in the transcription of the majority of cytokines investigated in aE/C- compared to E-/C+ chickens, despite considerable attenuation of the WisF96 parasite line. To the best of our knowledge this is the first report of immune responses associated with *in vivo* WisF96 infection. The induction of immune responses in the absence of significant pathology is relevant to the efficacy of attenuated anticoccidial vaccines. It is postulated that the reduction in *C. jejuni* colonisation in the liver and spleen in the co-infection model could be due to an associated, 'bystander' immune response induced by the parasite. *E. tenella* infection stimulates a strong pro-inflammatory immune response including significant increases in IFNγ and iNOS (39). iNOS has also been directly linked to the control of *C. jejuni* (40). Caecal iNOS transcription was increased six- or eight-fold during infection with attenuated or non-attenuated *E. tenella*. The up-regulation of immune factors linked to control of *C. jejuni* as a consequence of an ongoing *E. tenella* infection may explain, at least in part, the 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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694 **Figure legends**

695 **Figure 1.** (A-C) *C. jejuni* load in single or co-infected Light Sussex chickens (Trial 2).
696 Circle = count per bird (log_{10}). X = average count per treatment group (log_{10}). (A)
697 Caecal contents. (B) Liver. (C) Spleen. (D) Total log_{10} *E. tenella* oocyst output per
698 bird (circle) and average per group (X). (E-G) Relationship between *C. jejuni* load
699 and *E. tenella* oocyst output. Solid markers = non-attenuated *E. tenella*, hollow
700 markers = attenuated *E. tenella*. (E) Caecal contents. (F) Liver. (G) Spleen. (Key)
701 Group identifiers and experimental schedule. nE = non-attenuated *E. tenella*
702 Wisconsin, aE = attenuated *E. tenella* WisF96, C = *C. jejuni* 81-176. + =
703 administered. - = not administered, mock control. LD = limit of detection. Groups with
704 different superscript letters within plot indicate significant statistical differences.

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

709 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]

710 nE = non-attenuated *E. tenella* Wis, aE = attenuated *E. tenella* WisF96, C = *C. jejuni*, + = administered, - = not administered. [1]= 8

711 birds/group. Averages that were significantly different within each column are identified by a different superscript letter ($p < 0.05$).

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Table 2. *Campylobacter jejuni* and *Eimeria tenella* dose regimes and viable counts from single and co-infection of chickens in Trial 3.

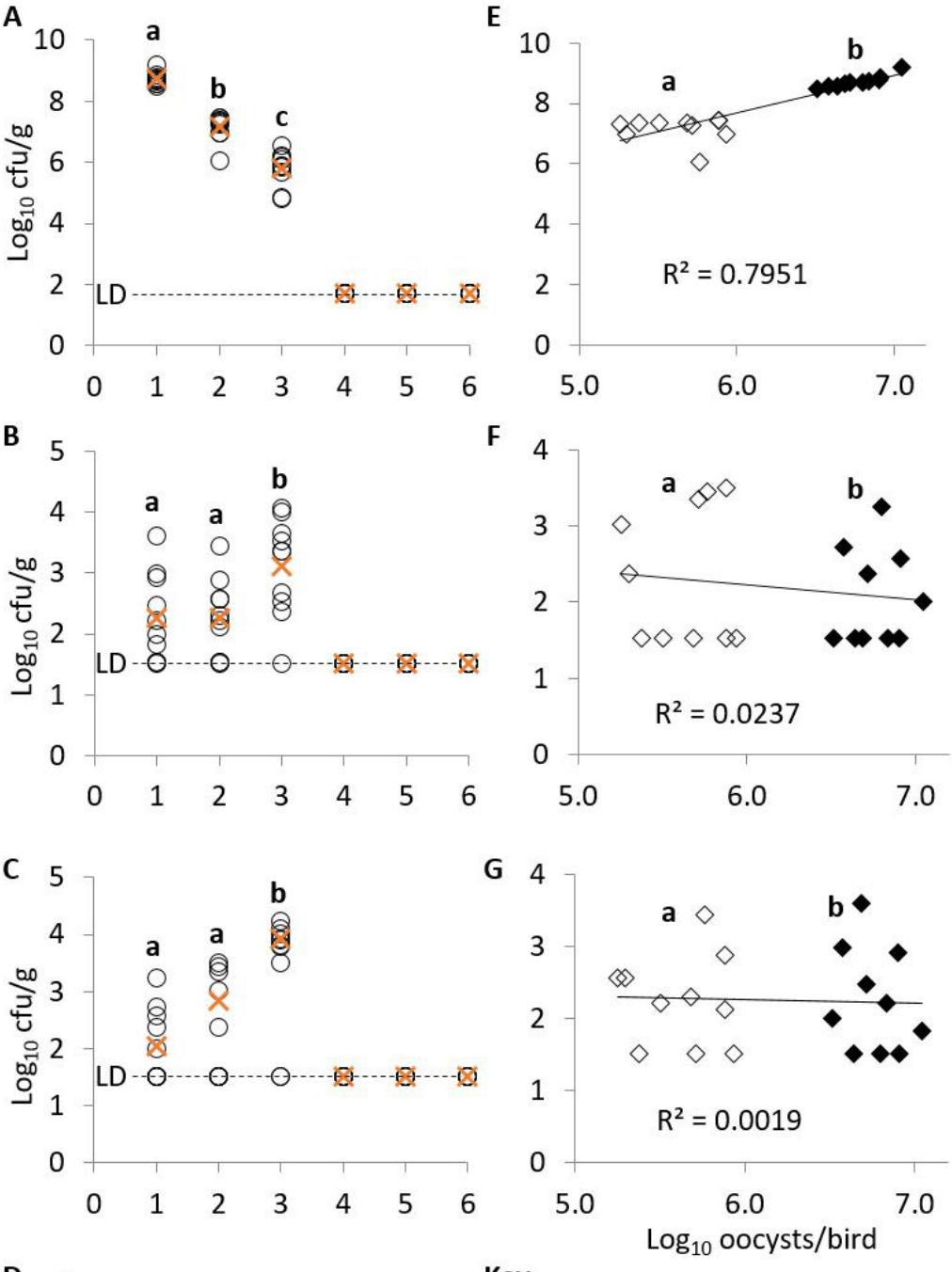
Group[1]	*E. tenella* strain (dose; age at dosing)	*C. jejuni* Log_{10} CFU (d18)	Log_{10} output oocysts per bird	Log_{10} CFU/g (Average ± SD)			
				Day 21[2]	Day 28[3]		
				Cloacal swab	Caeca	Liver	Spleen
nEh+/C+	Wis (4000; d13)	8.27	7.28 ± *0.06*^{a}	9.16 ± *0.51*^{a}	8.47 ± *0.51*^{a}	1.99 ± *0.19*^{a}	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

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

723 **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*

724 nE = non-attenuated *E. tenella* Wis, aE = attenuated *E. tenella* WisF96, C = *C. jejuni*, + = administered, - = not administered. Fold change data that were
725 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*
726 challenge.



Key

Group	*E. tenella* (dose, age at dosing)	*C. jejuni* (Log_{10} 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-	-	-