



# Impact of *Eimeria tenella* Coinfection on *Campylobacter jejuni* Colonization of the Chicken

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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 recognized; however, less is known about the secondary effects that infection may have on other gut pathogens. *Campylobacter jejuni* is the leading cause of human bacterial foodborne 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 the colonization and replication of bacteria, such as *Clostridium perfringens* and *Salmonella enterica* serovar Typhimurium. Through a series of *in vivo* coinfection experiments, this study evaluated the impact that *E. tenella* infection had on *C. jejuni* colonization of chickens, including the influence of variations in parasite dose and sampling time after bacterial challenge. Coinfection with *E. tenella* resulted in a significant increase in *C. jejuni* colonization in the cecum in a parasite dose-dependent manner but a significant decrease in *C. jejuni* colonization in the spleen and liver of chickens. The results were reproducible at 3 and 10 days after 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.

**KEYWORDS** *Campylobacter jejuni*, chicken, coinfection, *Eimeria tenella*

The 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 the 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 colonization and replication of pathogens in the chicken intestine, with a classic example being the enhanced growth of *Clostridium perfringens* potentiated by the high mucus production induced by coinfecting *Eimeria* species parasites (5). Recently, the translocation of *Escherichia coli* from the gut to internal organs was shown to be enhanced by coinfection with *Campylobacter jejuni* (6). Moreover, an extensive study of commercial broiler flocks showed a strong association between *Campylobacter* isolation and the 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 colonization by pathogenic bacteria, such as *C. perfringens* and *Salmonella enterica* serovar Typhimurium (5, 10). The

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**TABLE 1** *Campylobacter jejuni* and *Eimeria tenella* dose regimes and viable counts from single infection and coinfection of chickens in trial 1

Group <sup>a</sup>	<i>E. tenella</i> strain (oocyst dose; age at dosing [days])	No. of <i>C. jejuni</i> log <sub>10</sub> CFU at day 18	Avg ± SD no. of log <sub>10</sub> CFU/g at day 21 <sup>b</sup> (3 days post <i>C. jejuni</i> )		
			Cecum	Liver	Spleen
nE <sup>+</sup> /C <sup>+</sup>	Wis (4,000; 13)	8.17	9.13 ± 0.19 <sup>A</sup>	2.03 ± 1.22 <sup>A</sup>	1.67 ± 1.51 <sup>A</sup>
aE <sup>+</sup> /C <sup>+</sup>	WisF96 (115,000; 15)	8.17	7.55 ± 0.62 <sup>B</sup>	2.03 ± 1.23 <sup>A</sup>	1.35 ± 1.20 <sup>A</sup>
E <sup>-</sup> /C <sup>+</sup>	None	8.17	6.61 ± 1.77 <sup>C</sup>	2.91 ± 1.53 <sup>A</sup>	2.70 ± 1.71 <sup>A</sup>

<sup>a</sup>Eight birds were in each group. nE, nonattenuated *E. tenella* Wis; aE, attenuated *E. tenella* WisF96; C, *C. jejuni*; +, administered; -, not administered.

<sup>b</sup>Day 21 was 3 days after *C. jejuni* inoculation. Averages that were significantly different within each column are identified by a different superscript letter ( $P < 0.05$ ).

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*.

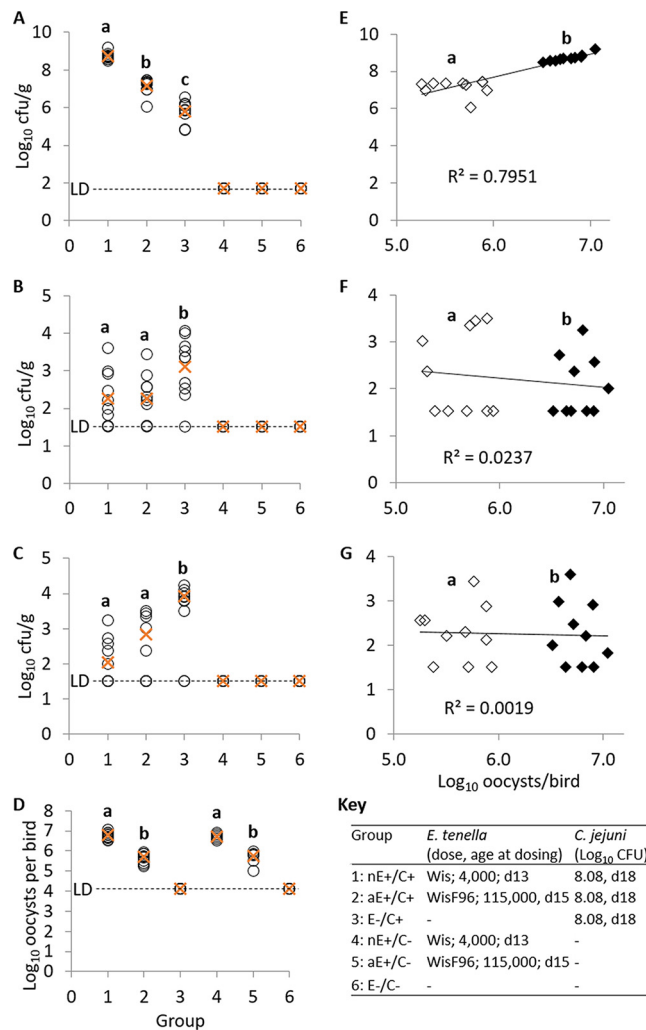
*C. jejuni* is the leading cause of human bacterial food poisoning in many countries, with an estimated global burden of 95 million illnesses and 21,000 deaths in 2010 and with 2.1 million disability-adjusted life years being lost in 2010 (13), and can induce severe sequelae, including inflammatory neuropathies, such as 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 the 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 2 to 3 weeks (18, 19). After this period, once the first bird becomes colonized, the infection spreads quickly throughout the flock via the fecal-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 than fecal surface contaminants to be destroyed by cooking (22). Increasingly, outbreaks of human campylobacteriosis are linked to the consumption of undercooked chicken products, such as liver pâté (23).

The aim of this study was to investigate the influence of concurrent *E. tenella* infection on *C. jejuni* colonization in chickens, including investigation of the physical and immunological factors associated with the observed changes. *E. tenella* causes hemorrhagic enteritis in the chicken cecum, accompanied by the induction of strong proinflammatory immune responses that include the 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 the immune responses and/or the pathology induced by *E. tenella* may allow *C. jejuni* to flourish and breach the protective gut wall, increasing colonization and replication within the cecum, liver, and spleen.

## RESULTS

***E. tenella*/C. *jejuni* coinfection.** For all 3 trials, at all sampling sites *C. jejuni* was not detected above the limit of detection in any of the unchallenged (C-) birds.

**(i) Trial 1.** In trial 1 (pilot study; Table 1), at 3 days after bacterial challenge, coinfection with nonattenuated or attenuated *E. tenella* caused a significant 2.5- or 1-log<sub>10</sub> increase in the *C. jejuni* load in the cecum ( $P < 0.001$  and  $P < 0.05$ ), respectively,



**FIG 1** (A to C) *C. jejuni* load in single or coinfecting Light Sussex chickens (trial 2). Circles, count per bird ( $\log_{10}$ );  $\times$ , average count per treatment group ( $\log_{10}$ ). (A) Cecal contents; (B) liver; (C) spleen. (D) Total  $\log_{10}$  *E. tenella* oocyst output per bird (circles) and average per group ( $\times$ ). (E to G) Relationship between *C. jejuni* load and *E. tenella* oocyst output. Solid symbols, nonattenuated *E. tenella*; hollow symbols, attenuated *E. tenella*. (E) Cecal contents; (F) liver; (G) spleen. Group identifiers and experimental schedule are abbreviated as follows: nE, nonattenuated *E. tenella* Wisconsin; aE, attenuated *E. tenella* WisF96; C, *C. jejuni* 81-176; +, administered; -, not administered (mock-infected control); LD, limit of detection. Different superscript letters within a plot indicate statistically significant differences between groups.

compared to that with infection with *C. jejuni* alone. A significant difference in cecal *C. jejuni* colonization was also detected between the nonattenuated and attenuated parasite-infected groups ( $P < 0.05$ ). In the spleen, coinfection with either of the *E. tenella* lines caused a nonsignificant 1- $\log_{10}$  decrease in the *C. jejuni* load ( $P > 0.05$ ) compared to that with infection with *C. jejuni* alone. Similarly, in the liver, coinfection with either parasite line caused a nonsignificant  $\sim 1$ - $\log_{10}$  decrease in the *C. jejuni* load ( $P > 0.05$ ).

**(ii) Trial 2.** In the cecum, at 3 days after bacterial challenge, coinfection with nonattenuated or attenuated *E. tenella* caused a significant 2.9- or 1.35- $\log_{10}$  increase in the *C. jejuni* load, respectively, compared to that with infection with *C. jejuni* alone ( $P < 0.001$ ; Fig. 1A). A significant difference in *C. jejuni* colonization was again detected in the cecum between the nonattenuated and attenuated parasite-infected groups ( $P < 0.001$ ). Here, the *C. jejuni* load was positively correlated with parasite replication, measured in terms of total oocyst output ( $r = 0.893$ ,  $P < 0.001$ ; Fig. 1E). In the liver,

**TABLE 2** *Campylobacter jejuni* and *Eimeria tenella* dose regimes and viable counts from single and coinfection of chickens in trial 3<sup>a</sup>

Group <sup>b</sup>	<i>E. tenella</i> strain (oocyst dose; age at dosing [days])	No. of <i>C. jejuni</i> log <sub>10</sub> CFU at day 18	Output (no. of log <sub>10</sub> oocysts/bird) <sup>c</sup>	Avg ± SD no. of log <sub>10</sub> CFU/g <sup>c</sup>			
				Day 21, <sup>d</sup> cloacal swab	Day 28 <sup>e</sup>		
				Cecum	Liver	Spleen	
nEh <sup>+</sup> /C <sup>+</sup>	Wis (4,000; 13)	8.27	7.28 ± 0.06 <sup>A</sup>	9.16 ± 0.51 <sup>A</sup>	8.47 ± 0.51 <sup>A</sup>	1.99 ± 0.19 <sup>A</sup>	2.42 ± 0.50 <sup>A</sup>
nEl <sup>+</sup> /C <sup>+</sup>	Wis (400; 13)	8.27	6.75 ± 0.09 <sup>B</sup>	7.64 ± 0.49 <sup>B</sup>	7.05 ± 0.93 <sup>B</sup>	2.72 ± 0.26 <sup>AB</sup>	2.60 ± 0.47 <sup>A</sup>
E <sup>-</sup> /C <sup>+</sup>	None	8.27	ND	7.56 ± 0.54 <sup>B</sup>	6.97 ± 1.03 <sup>B</sup>	3.06 ± 0.32 <sup>B</sup>	3.27 ± 0.82 <sup>A</sup>
nEh <sup>+</sup> /C <sup>-</sup>	Wis (4,000; 13)	Mock	7.28 ± 0.04 <sup>A</sup>	ND	ND	ND	ND
nEl <sup>+</sup> /C <sup>-</sup>	Wis (400; 13)	Mock	6.73 ± 0.07 <sup>B</sup>	ND	ND	ND	ND
E <sup>-</sup> /C <sup>-</sup>	None	Mock	ND	ND	ND	ND	ND

<sup>a</sup>nE, nonattenuated *E. tenella* Wis; C, *C. jejuni*; h, high dose; l, low dose; +, administered; -, not administered; Mock, no bacterial control; ND, none detected.

<sup>b</sup>Eight birds were in each group.

<sup>c</sup>Averages that were significantly different within each column are identified by a different superscript letter ( $P < 0.05$ ).

<sup>d</sup>Sampled 3 days after *C. jejuni* inoculation.

<sup>e</sup>Sampled 10 days after *C. jejuni* inoculation.

coinfection with nonattenuated and attenuated *E. tenella* caused a significant  $\sim 1$ -log<sub>10</sub> decrease in the *C. jejuni* load ( $P < 0.05$ ; Fig. 1B), although no difference between the parasite lines was detected ( $P > 0.05$ ). Similarly, in the spleen, coinfection with the nonattenuated and attenuated *E. tenella* line caused significant 1.8- and 1.1-log<sub>10</sub> decreases in the *C. jejuni* load, respectively ( $P < 0.05$ ; Fig. 1C), with no difference between the parasite lines being detected. In both the liver and spleen, no association between the *C. jejuni* load and the level of fecal oocyst output was detected ( $P > 0.05$ ; Fig. 1F and G). The total oocyst output was higher in chickens infected with nonattenuated *E. tenella* than in those infected with the attenuated line (Fig. 1D).

**(iii) Trial 3.** In trial 3 (Table 2), cloacal swab specimens were collected from all groups at 3 days after *C. jejuni* infection. Coinfection initiated with a high nonattenuated *E. tenella* dose caused a significant, 1.6-log<sub>10</sub> increase in the cloacal *C. jejuni* load ( $P < 0.001$ ) compared to that with infection with *C. jejuni* alone. In the coinfecting group infected with a low parasite dose, no difference in the *C. jejuni* load from that in the group infected with *C. jejuni* alone was observed ( $P > 0.05$ ). A significant difference in the cloacal *C. jejuni* load was noted between the groups coinfecting with high and low parasite doses ( $P < 0.001$ ).

In the cecum, at 10 days after *C. jejuni* infection, coinfection initiated with a high *E. tenella* dose caused a significant 1.5-log<sub>10</sub> increase in *C. jejuni* colonization compared to that with infection with *C. jejuni* alone ( $P < 0.01$ ). There was a significant association with oocyst output ( $r = 0.682$ ,  $P = 0.001$ ). Coinfection with the low parasite dose did not cause a significant change in *C. jejuni* colonization compared to that with *C. jejuni* alone ( $P < 0.05$ ). A significant variation in the level of *C. jejuni* colonization was noted between the groups infected with high and low doses of *E. tenella* ( $P < 0.01$ ).

In the spleen, at 10 days after *C. jejuni* infection, no significant difference in the levels of *C. jejuni* in the presence and in the absence of *E. tenella* was detected; however, a nonsignificant ( $P > 0.05$ ) decreasing trend in *C. jejuni* colonization was observed. No association between *C. jejuni* colonization in the spleen and the level of fecal oocyst output was detected ( $r = -0.44$ ,  $P > 0.05$ ).

In the liver, at 10 days after *C. jejuni* infection, there was a significant decrease in *C. jejuni* colonization in the group infected with a high dose of *E. tenella* compared to that in the group infected with *C. jejuni* alone ( $P < 0.05$ ). No significant changes from the group infected with a low parasite dose were observed. No association between the *C. jejuni* load in the liver and the level of fecal oocyst output was detected ( $r = -0.31$ ,  $P > 0.05$ ).

**Cytokine response to *E. tenella*/*C. jejuni* challenge.** *E. tenella* infection induces a strong immune response, and it was postulated that the changes in the *C. jejuni* load noted in the coinfection models could be due to an associated bystander immune response. Cecal tissues collected during trial 2 at 21 days of age were used to investigate the transcription of a variety of cytokines (i.e., at a single time point, equivalent to

**TABLE 3** Transcriptional fold change of cytokines and mucins in cecal tissue collected during trial 2<sup>a</sup>

Target gene	Fold change ± SEM versus <sup>b</sup> :						
	Uninfected birds					<i>C. jejuni</i> only-infected group	
	nE <sup>+</sup> /C <sup>+</sup>	aE <sup>+</sup> /C <sup>+</sup>	E <sup>-</sup> /C <sup>+</sup>	nE <sup>+</sup> /C <sup>-</sup>	aE <sup>+</sup> /C <sup>-</sup>	nE <sup>+</sup> /C <sup>+</sup>	aE <sup>+</sup> /C <sup>+</sup>
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
IFN-γ	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

<sup>a</sup>Samples were collected 3 days after *C. jejuni* challenge. nE, nonattenuated *E. tenella* Wis; aE, attenuated *E. tenella* WisF96; C, *C. jejuni*; +, administered; -, not administered.

<sup>b</sup>Fold change data that were significantly different are identified by asterisks, as follows: \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ . ns, not significant.

7, 5, and 3 days after challenge by the Wisconsin [Wis] strain, the WisF96 strain, and *C. jejuni*, respectively). The fold change in the level of transcription in each group compared to that in the uninfected control group is summarized in Table 3, along with the fold change in the coinfecting groups from that in the group infected with *C. jejuni* only. Infection with *C. jejuni* alone significantly increased the transcription of interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) ( $P \leq 0.001$  for both), as well as IL-13 ( $P \leq 0.01$ ). Infection with nonattenuated or attenuated *E. tenella* increased the cecal transcription of IL-1β, IL-2, IL-6, IL-10, iNOS, and gamma interferon (IFN-γ) significantly compared to that in the uninfected and *C. jejuni*-only-infected groups, irrespective of *C. jejuni* coinfection. Transcription of IL-13 was significantly decreased in all *Eimeria*-infected groups. The accompanying *P* values are indicated in Table 3.

**Mucin gene transcription in response to *E. tenella*/*C. jejuni* challenge.** Cecal 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 at 3 days postchallenge (Table 3). Infection with nonattenuated *E. tenella* resulted in the upregulation of *muc2*, *muc5ac*, and *muc13* transcription, most notably, the transcription of *muc5ac*, which was the only *muc* gene significantly upregulated during attenuated *E. tenella* infection.

## DISCUSSION

*In vivo* trials were carried out to analyze the impact of parasite coinfection on *C. jejuni* colonization of the cecum, spleen, and liver of chickens. The local transcription of selected cytokine and mucin genes was assessed in an effort to explain the differences detected. It was hypothesized that damage to the cecal epithelial barrier induced by the hemorrhagic parasite *E. tenella* and/or the consequential proinflammatory immune response would facilitate increased bacterial colonization in the cecum, liver, and spleen. Quantification of *C. jejuni* colonization 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 coinfection was associated with elevated *C. jejuni* loads within the cecal contents but reduced loads in the liver and spleen. Thus, while fecal 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 coinfecting with *Eimeria* parasites and either *C. perfringens* or *S. enterica* serovar Typhimurium (5, 10). It has been shown that *E. tenella* infection can influence the cecal microflora in a manner that has been reported by some (28, 29) to potentially benefit *C. jejuni* colonization, which demonstrates that *E. tenella*-induced dysbiosis may increase susceptibility to enteric pathogens, such as *C. jejuni*. Further analysis of the microbiome of coinfecting poultry is needed to investigate this hypothesis. An increased bacterial load in the gut but not the internal organs due

to coinfection 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 utilize transgenic attenuated strains of the parasite to deliver vaccine antigens to chickens. Live attenuated vaccines are currently used to vaccinate over 1 billion birds each year (11), and results from this study suggest that attenuated strains have the potential to reduce *C. jejuni* colonization in the liver of poultry, which could limit human cases of campylobacteriosis. Paradoxically, increases in *C. jejuni* colonization in the cecum are of concern, although improvements in abattoir protocols have been associated with a shift from the importance of surface contamination by feces to the importance of deep tissue colonization by *C. jejuni*, exacerbated by the deliberate undercooking or sautéing of chicken liver due to the belief that this will enhance the flavor and appearance of the end product (30).

It is well recognized that, individually, both *E. tenella* and *C. jejuni* generate an immune response of varying levels in chickens following infection (24, 27, 31–33). The impact of *E. tenella* coinfection on *C. jejuni* colonization and the concurrent effect on cytokine production have not been reported. Previously wild-type (nonattenuated) 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 (31, 32). These findings were replicated in this study, where the transcription of all but one of the cytokines tested, IL-13, was increased in nonattenuated *E. tenella* Wis-infected, *C. jejuni*-uninfected ( $nE^+/C^-$ ) chickens compared to *E. tenella*-uninfected, *C. jejuni*-infected ( $E^-/C^+$ ) chickens. Additionally, in this study it is notable that there was a significant increase in the transcription of the majority of cytokines investigated in attenuated *E. tenella* WisF96-infected, *C. jejuni*-uninfected ( $aE^+/C^+$ ) chickens compared to  $E^-/C^+$  chickens, despite the 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* colonization in the liver and spleen in the coinfection model could be due to an associated, bystander immune response induced by the parasite. *E. tenella* infection stimulates a strong proinflammatory immune response, including significant increases in IFN- $\gamma$  and iNOS transcription (34). iNOS has also been directly linked to the control of *C. jejuni* (35). The level of cecal iNOS transcription was increased 6- or 8-fold during infection with attenuated or nonattenuated *E. tenella*. The upregulation of immune factors linked to the 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 coinfecting chickens. IFN- $\gamma$  levels are balanced by anti-inflammatory cytokines, such as IL-10 (36). Humphrey et al. (2014) reported that the regulation of IL-10 is important in controlling intestinal pathology in *C. jejuni*-infected chickens, where lower levels are associated with prolonged inflammation and diarrhea (31). In support of that finding, Vaezirad et al. (2017) demonstrated that using glucocorticoids to dampen the immune system of chickens reduced the expression of proinflammatory genes and increased the colonization of *C. jejuni* in the cecum as well as translocation to and colonization of the liver (37). The work of Vaezirad et al. (37) supports the hypothesis that the increase in *C. jejuni* cecal colonization may also be influenced by physical damage. *E. tenella* infection causes the sloughing of cells which form the epithelial barrier, and this damage may facilitate enhanced *C. jejuni* colonization in the cecum, akin to the mechanism utilized by *C. perfringens* to invade the gut in the presence of *Eimeria* (38, 39).

Increased transcription of the majority of cytokines in the cecal tissue in coinfecting birds did not appear to impede *C. jejuni* colonization of the cecal contents, although it is not clear if this was a cause or an effect. These results suggest that the mechanism(s) responsible for the increase in *C. jejuni* colonization detected within the cecal lumen is distinct from translocation through the cecal wall and/or deep tissue colonization. *E. tenella* can cause a hemorrhagic form of coccidiosis characterized by large volumes of

blood in the cecum (40). Iron is an essential nutrient for the colonization of *C. jejuni*; however, bioavailability is limited within many host environments (41). Bacteria can take up iron via environmental sources, such as hemin and hemoglobin (42). It is hypothesized that the increased availability of hemoglobin in the cecum, due to the 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 nonattenuated and attenuated parasite infections supports such a hypothesis, and it is noted that the attenuated line was expected to induce little or no hemorrhage. Attenuated *E. tenella* parasites are less pathogenic than nonattenuated parasites (43) and cause less damage to the intestinal epithelium, but they still induce an immune response equivalent to that induced by nonattenuated parasites (44). The subsequent comparison of high and low nonattenuated parasite doses confirmed a dose-effect of *Eimeria* on *C. jejuni* colonization within the cecal contents but not the liver or spleen, supporting the association between pathological severity and dose in the former but not the latter. While the parasite crowding effect is expected to have reduced the scale of the difference between the high and low doses by the time of oocyst excretion (45), it is clear that pathology (lesion score) does associate with dose level (46). Variations in unidentified immune factors may contribute to this effect and could influence the increased cecal *C. jejuni* load in chickens coinfecting with the attenuated parasite, where cecal pathology would have been minimal.

Trials 1 and 2 explored the impact of an ongoing infection with nonattenuated or attenuated *E. tenella* parasites on *C. jejuni* colonization of chickens 3 days after bacterial challenge. The healthy chicken cecum empties several times per day, suggesting that the figures recorded represent true bacterial colonization (47). However, to confirm the association, the study was repeated using a later sampling point, revealing results at 10 days similar to those obtained at 3 days after bacterial challenge. Once *C. jejuni*-contaminated food or fecal material is ingested by the chicken, the transit time through the upper gastrointestinal tract is ~2.5 h (48). Work by Shaughnessy et al. (2009), using an inoculating dose similar to the doses used in this study, showed high levels of persistent cecal colonization at 6, 20, and 48 h after *C. jejuni* infection, indicating rapid colonization of the bacteria in the cecum (33). Meade et al. (2009) showed that the liver and spleen of the majority of birds were colonized by *C. jejuni* at 48 h postinfection (49). These studies support analysis of *C. jejuni* colonization in the *E. tenella* coinfection model at 3 days after bacterial infection, and the results should be confirmed at 10 days postinfection. Practically, these results are also relevant to the field situation, where anticoccidial drugs are commonly withdrawn from broiler diets 3 to 5 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 hemorrhage, 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 colonization, including flagellin A and *Campylobacter* invasion antigens, are known to be secreted in the presence of chicken mucus (50, 51). Chicken mucus has also been shown to enhance *C. jejuni* motility and expression of the flagellar protein FlgR (52), to protect *C. jejuni* from some short- and medium-chain fatty acids (53, 54), and the viscous environment might aid binding and invasion of mammalian cells (55). However, enteric mucus from chickens has also been reported to attenuate *C. jejuni* 81-176 invasion of both avian and human epithelial cells (56), possibly contributing to reduced translocation away from the cecum. Mucins are a major component of mucus, and in this study, the transcription of *muc2* and *muc5ac* (both of which are secreted, mucus-forming mucins [57]) and *muc13* (a transmembrane mucin) increased in the presence of nonattenuated *E. tenella*. The 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 colonization 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) (58, 59) was carried out during an *in vivo* coinfection trial to test this theory (summarized in the Methods section and Table S2 in the supplemental material). It was hypothesized 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 cecal lumen and indirectly reducing *C. jejuni* replication and colonization. Further, depleting the secreted mucus layer might be expected to facilitate increased translocation to extraintestinal sites, such as the liver and spleen. In mucin 2-deficient mice presenting with a diminished intestinal barrier, the levels of infection and mortality caused by *S. enterica* serovar Typhimurium were increased (60). 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 the cecal *C. jejuni* load, with a further nonsignificant reduction in treated compared to untreated single *C. jejuni*-infected chickens being seen. These results support the view that chicken mucus may aid *C. jejuni* colonization and/or replication, possibly via the provision of nutrients required for sustained growth (61), 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 in *C. jejuni* colonization of the liver and spleen detected in the coinfection model were maintained in the presence of NAC, suggesting either a limited role for mucus in this aspect of the parasite-bacterium interaction or the inefficacy of the NAC protocol.

**Conclusion.** The current study has demonstrated that *E. tenella* coinfection exerts a significant impact on colonization by *C. jejuni* in Light Sussex chickens, while it upregulates several relevant immune factors. Coinfection caused a significant increase in *C. jejuni* colonization in the cecal contents in a parasite pathology- and dose-dependent manner but a decrease in the liver and spleen. The results were reproducible on days 3 and 10 after 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 coinfection studies with broiler chickens raised under intensive farming 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* colonization of poultry may impact both the use of live anticoccidial vaccines and the development of *Eimeria* as a novel vaccine vector.

## 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 the protocols being approved by the Institute for Animal Health and the Royal Veterinary College (RVC) 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 license 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 to be specific pathogen free (SPF). Throughout the study, all chickens had access to food and water *ad libitum* and were fed 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 (62, 63). The Wis strain is a wild-type (nonattenuated) *E. tenella* isolate with a standard prepatent period of ~132 h. The WisF96 line has been attenuated by selection for precocious development, resulting in a single round of schizogony with a reduced prepatent period of ~96 h and much reduced pathology due to the loss of the second-generation schizont, which is responsible for deep tissue damage and hemorrhage (63). Nonetheless, the WisF96 line retains the ability to induce a fully protective immune response during natural infection that is comparable to that induced by the nonattenuated Wis strain (62). These parasites are phenotypically stable, were passaged through chickens at the Institute for Animal Health and then the Royal Veterinary College through dosing and recovery as previously described (64), and were used in these studies less than 1 month after sporulation.

**Bacterial propagation.** *C. jejuni* strain 81-176 was used due to its proven ability to efficiently colonize the chicken gastrointestinal tract (65). Bacteria were routinely cultured in Mueller-Hinton (MH)



broth and on sheep blood agar plates at 37°C for 48 h in a microaerophilic atmosphere created using the CampyGen system (all from Oxoid, Basingstoke, UK). Charcoal cefoperazone deoxycholate agar (CCDA; Oxoid) was used to retrospectively enumerate the CFU 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 the *C. jejuni* bacteria recovered from chickens by directly plating 10-fold serial dilutions of homogenates of cecal contents, liver, and spleen (as described below). The plates were incubated at 37°C for 48 h in a microaerophilic atmosphere, as detailed above. Animals not challenged using *C. jejuni* were screened for exposure to *Campylobacter* by enrichment of cecal contents using modified Exeter broth as described previously (65), followed by plating on CCDA plates.

**Experimental design. (i) *E. tenella/C. jejuni* coinfection.** 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 3 groups of eight. Chickens in group 1 received 4,000 sporulated *E. tenella* Wis (nonattenuated) oocysts by oral gavage at 13 days of age (nE<sup>+</sup>). Chickens in group 2 received 115,000 sporulated *E. tenella* WisF96 (attenuated) oocysts by oral gavage at 15 days of age (aE<sup>+</sup>). Chickens in group 3 were not infected with *E. tenella*. Chickens in all 3 groups received ~10<sup>8</sup> CFU of *C. jejuni* by oral gavage at 18 days of age (C<sup>+</sup>). The differential dosing schedule of nE<sup>+</sup>/C<sup>+</sup> chickens and aE<sup>+</sup>/C<sup>+</sup> chickens was to adjust for the different prepatent periods of these parasites, to ensure peak parasitemia in the ceca at the time of *C. jejuni* challenge in both groups. The nonattenuated and attenuated parasite lines were used to compare the severity of the pathology (i.e., the presence or 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 an equivalent oocyst output was not expected (62). Parasite-associated pathology was anticipated only for the nonattenuated Wis-infected groups. At 3 days after *C. jejuni* challenge (21 days of age), all birds were culled. Postmortem cecal contents and liver and spleen tissue were collected immediately.

Trial 2 followed an experimental outline similar to that for trial 1, with groups 1 to 3 (nE<sup>+</sup>/C<sup>+</sup>, aE<sup>+</sup>/C<sup>+</sup>, and *E. tenella*-uninfected and *C. jejuni*-infected [E<sup>-</sup>/C<sup>+</sup>] chickens, respectively) receiving identical treatment (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 to 6; *E. tenella* Wis only-treated [nE<sup>+</sup>/C<sup>-</sup>], *E. tenella* WisF96 only-treated [aE<sup>+</sup>/C<sup>-</sup>], and *E. tenella*- and *C. jejuni*-untreated [E<sup>-</sup>/C<sup>-</sup>] chickens, respectively). Groups 1 to 3 (all C<sup>+</sup>) comprised 10 Light Sussex chickens per group, while groups 4 to 6 (all C<sup>-</sup>) comprised six chickens per group, reflecting the greater bird-to-bird variation in *C. jejuni* enumeration than in *E. tenella* enumeration. All birds were caged separately to facilitate collection of individual bird feces and enumeration of the total daily oocyst output between 18 and 21 days of age, as described previously (66). All birds were culled at 3 days after *C. jejuni* challenge (21 days of age), and samples were collected as described above 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 nonattenuated *E. tenella* Wis was used to assess the effect of parasite dose/replication rather than the effect of reduced pathogenicity on the outcome of *C. jejuni* infection. In this trial, the culling of birds was delayed to 10 days after *C. jejuni* challenge to assess if the changes observed in the *C. jejuni* load at 3 days (trials 1 and 2) were stable over a longer period. Additionally, to provide a semiquantitative comparison of the bacterial load between trials 1, 2, and 3, birds were swabbed cloacally at 3 days after *C. jejuni* challenge, as described previously (11). At 13 days of age, groups 1 and 4 received a high dose of 4,000 sporulated *E. tenella* Wis oocysts (nEh<sup>+</sup>/C<sup>+</sup> and nEh<sup>+</sup>/C<sup>-</sup> chickens, respectively), while groups 2 and 5 received a low dose of 400 sporulated *E. tenella* Wis oocysts (nEl<sup>+</sup>/C<sup>+</sup> and nEl<sup>+</sup>/C<sup>-</sup> chickens, respectively). Chickens in groups 3 and 6 were not infected with the parasite (E<sup>-</sup>/C<sup>+</sup> and E<sup>-</sup>/C<sup>-</sup> chickens, respectively). At 18 days of age, groups 1, 3, and 5 were challenged with ~10<sup>8</sup> CFU of *C. jejuni*, while groups 2, 4, and 6 were mock challenged with sterile MH broth. The daily oocyst output was assessed for each chicken at between 18 and 22 days of age. Chickens were culled at 10 days after bacterial challenge (28 days of age), and samples were collected as described above for trial 1.

**(ii) Sample collection.** Postmortem, 0.2 to 1.0 g of cecal contents and liver and spleen tissue was collected aseptically from the same approximately central part of each tissue/organ, placed into universal tubes, and stored separately on ice prior to homogenization in all trials. On the day of collection, all samples were weighed and homogenized in an equal volume (wt/vol) of sterile PBS using a TissueRuptor homogenizer (Qiagen, Hilden, Germany), followed by serial 10-fold dilution in PBS. Additionally, an ~3-cm tissue section from the midpoint of one cecum, half the spleen, and an ~1-cm<sup>3</sup> section of the mid-liver were recovered from chickens in trial 2 and stored in RNAlater stabilization solution (Sigma), as recommended by the manufacturer, for subsequent RNA extraction and real-time quantitative PCR (RT-qPCR).

**RNA extraction and integrity.** Total RNA was extracted from thawed tissue samples after storage at -20°C in RNAlater stabilization solution using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The optional DNase digestion step was included to remove contaminating genomic DNA. The RNA concentration was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and samples were diluted in nuclease-free water to produce a final concentration of 40 ng/μl. The quality of a subset 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 6 for further analysis.

**RT-qPCR.** SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used to make cDNA, using total RNA purified from the samples collected, following the manufacturer's instructions. Oligo-(dT)<sub>12-18</sub> (Invitrogen, Carlsbad, CA, USA) was used along with the optional RNaseOut recombinant ribonuclease inhibitor (Invitrogen) step. cDNA was used as the template in all RT-PCRs.

The oligonucleotide primer sequences used to target the cDNA copies of each of the mRNA transcripts investigated, including those for the genes for Muc2, Muc5ac, Muc13, IL-1 $\beta$ , IL-6, IFN- $\gamma$ , IL-2, IL-10, IL-13, and inducible nitric oxide synthase (iNOS), and three reference transcripts are summarized in Table S1 in the supplemental material. The final reaction volumes for RT-qPCR consisted of 10  $\mu$ l SsoFast EvaGreen super mix containing Sybr Green dye (Bio-Rad) and 70 nM each forward and reverse primer (Sigma-Aldrich) and were made up to 19  $\mu$ l using RNase- and DNase-free water (Invitrogen, Paisley, UK). To 1 volume of this master mix, 1  $\mu$ l of cDNA was added. As a negative control, 1  $\mu$ l of water was used in place of cDNA. DNA was amplified on a Bio-Rad CFX (v2.0) cyclor (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 Table S1) 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. The levels of the individual transcripts were normalized individually to those of the transcripts of the 3 reference genes and used to calculate a mean value for each replicate. Briefly, quantification cycle ( $C_q$ ) values were generated for each sample using Bio-Rad CFX (v2.0) software and enabled quantification of cDNA when normalized to the values for the reference genes, consisting of the genes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TATA-BP), and 28S rRNA.

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

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00772-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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