Probiotic *Bacillus amyloliquefaciens* H57 ameliorates subclinical necrotic enteritis in broiler chicks by maintaining intestinal mucosal integrity and improving feed efficiency

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ABSTRACT Subclinical necrotic enteritis (**NE**) was induced in broiler chicks using a high dose of *Eimeria* spp. vaccine in the drinking water on day 9, and *Clostridium perfringens* (***Cp***) culture mixed in the feed on days 14 and 15. The aim was to evaluate the effects of probiotic *Bacillus amyloliquefaciens* strain H57 (**H57**) in preventing NE in chicks. Day-old Ross 308, male broilers were weighed and randomly assigned to 6 treatment groups (6 replicate cages/treatment and 8 birds/cage). Birds in group 1 (control) were fed the basal wheat-soybean diet without H57 or NE infection; in group 2 (*Eimeria*) were treated with *Eimeria* alone; in group 3 (*Cp*) were treated with *Cp* alone; in group 4 (NE) received both *Eimeria* and *Cp*; in group 5 (NE-H57) received NE infection and H57; and group 6 (H57) received H57. The basal diet of chicks in groups 5 and 6 was supplemented with H57 at a density of 2×10^8 spores/g feed from 1 D of age. On day 21, there were no significant treatment effects on BW and feed intake between control and H57 birds. However, on day 21, the feed conversion ratio of NE-H57 birds was significantly improved when compared with NE birds (1.28 vs. 1.36; $P < 0.001$). Birds challenged with NE had a higher occurrence of pasty vent than birds infected with either *Eimeria*, *Cp*, or NE-H57 (41 vs. 27 vs. 29 vs. 19%, respectively; $P < 0.001$). Intestinal lesion scores of NE birds were also higher than those of *Eimeria*, *Cp*, and NE-H57 birds (5.67 vs. 2.56 vs. 2.78 vs. 2.10, respectively; $P < 0.001$) and correlated with pasty vent (Pearson's r = 0.56; $P < 0.001$). Microscopic evaluation showed mucosal damage and necrosis in NE birds. In contrast, villi from NE-H57 birds were normal, with no damage or infiltration with *Eimeria* or *Cp*. H57 appears to be effective in challenged birds, as it maintained epithelial barrier integrity and improved feed efficiency.

Key words: broiler, probiotic, necrotic enteritis, intestinal mucosal integrity, feed efficiency

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INTRODUCTION

The use of antibiotics for improved performance efficiency of meat chickens has reduced dramatically globally, and in some countries, it is explicitly banned. Hence, the quest for antibiotic replacements and probiotics are showing promise (Bajagai et al., 2016). More evidence is required to validate the beneficial effects of probiotics. Previous broiler growth studies with *Bacillus amyloliquefaciens* strain H57 (**H57**) gave variable bird performance (Bajagai, 2018). However, in these experimental trials, birds were assessed under what would be considered optimal conditions, where most stressors were removed from the environment. There is no doubt that broilers or meat chickens grown commercially are subject to a complex of environmental stressors, especially in early life, which can impact bird health and performance. There is some evidence that probiotics perform better when a bird or animal is under stress from infectious, nutritional, or thermal stressors (Sohail et al., 2010; Abdelrahman et al., 2014; Cengiz et al., 2015; Martin Manuel et al., 2017), rather than in an optimized experimental setting.

Enteric infections are important environmental challenges for the broiler industry, and necrotic enteritis (**NE**) has become one of the most significant broiler

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diseases globally (Broom, 2017; Jones et al., 2019). It is associated with high economic loss owing to decreased growth and feed efficiency, increased morbidity/mortality rates, and high costs of control strategies, especially medication (Timbermont et al., 2011). Experimental induction of NE in chickens has been used as a tool to study the pathogenesis of enteric diseases and also to assess the efficacy of antimicrobials and alternative gut health therapeutics (Moore, 2016). In broiler chickens, *Clostridium perfringens* (***Cp***) is the major etiologic agent for NE diseases (Parish, 1961; Long and Truscott, 1976; Kaldhusdal and Hofshagen, 1992). There are other predisposing factors, which alter the microenvironment of the gastrointestinal tract (**GIT**) and promote *Cp* growth and colonization. Most NE models expose birds to a dual-infection system, involving *Eimeria* spp. and a pathogenic *Cp* strain isolated from NE outbreaks (Al-Sheikhly and Al-Saieg, 1980; Gholamiandehkordi et al., 2007; Park et al., 2008). However, a number of investigators have successfully produced disease without the use of coinfection (Cooper and Songer, 2010; Keyburn et al., 2010; Smyth and Martin, 2010). In some models, an immunosuppressive vaccine (e.g., Marek's disease virus, infectious bursal disease virus, and chicken anemia virus) is used before the infection with *Cp* (McReynolds et al., 2004; Hoerr, 2010).

Some dietary factors also influence NE development (Riddell and Kong, 1992; Kaldhusdal and Skjerve, 1996; Wu et al., 2014), and each could exert multiple effects (Moore, 2016). For example, the addition of fish meal to the diet of chickens alters intestinal gut microbiota and provides high nutrient levels for *Cp* growth. It has been suggested that fish meal diets cause cell damage from biogenic amines and also act as a source of infection with *Cp* (Wu et al., 2010, 2014; Rodgers et al., 2015). Feedstuffs containing high amounts of water-soluble nonstarch polysaccharides, such as barley, rye, and wheat, increase digesta viscosity and thereby GIT residence time and provide complex carbohydrates that can support proliferation of *Cp* (Kaldhusdal and Hofshagen, 1992; Annett et al., 2002; Jia et al., 2009).

In the present study, the effects of the probiotic H57 on the health and performance of broiler chicks reared in a stressful environment were investigated. The aim was 2-fold, to establish a subclinical NE model in the broiler chick, and assess the efficacy of H57 in improving intestinal health and preventing NE in birds. It was our hypothesis that H57 spores administered in the diet would maintain GIT health and improve feed utilization during a NE challenge.

MATERIALS AND METHODS

Animal Ethics Statement

The experimental studies and procedures involving meat chickens were approved by the Animal Ethics Committee of the University of Queensland (SAFS/192/18), as required by The Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Birds, and Bird Management

A total of 288 1-day-old male broiler chicks (Ross 308) were obtained from a commercial breeder (Aviagen, Australia Pty Ltd.). Birds were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease at the hatchery. From day 1, birds were placed in cages at a stocking density of 13 birds/m^2 and kept in an isolated, temperature controlled room at the poultry research facility on Gatton Campus (University of Queensland). The facility was thoroughly disinfected before bird placement. On arrival, chicks were individually weighed and allocated to 1 of 48 cages by stratified randomization so that each cage of chicks had the same initial mean and range of live weights. There were 6 groups (treatments), each containing 6 replicate cages of 8 birds per replicate/cage. The room temperature was gradually decreased, from 33°C on day 1 to 23°C on day 21. Birds were kept in a 24-h light program. From day 1, birds were fed an all-phase control wheat and soybean–based mash diet with or without added H57 (Table 1), till the end of the trial (day 21). Feed and water was supplied ad libitum, unless otherwise stated in the experimental protocol, during treatments (refer to day 9 and 14 on Table 2). Strict hygienic and biosecurity management practices were followed to prevent cross contamination between control and treated birds (i.e., challenged vs. unchallenged and H57-treated vs. H57-untreated birds) by keeping the treatment units apart and handling control birds first for all procedures (e.g., feeding, weighing, and sampling).

Experimental Design and Diets

The trial comprised 6 treatment groups that are detailed in Table 2. Briefly, group 1 represented the negative control (**control**), and birds were not treated with *Cp* or with *Eimeria* spp. vaccine and not fed a diet supplemented with H57. The trial also included 2 positive control groups: group 2 birds treated with *Eimeria* spp. vaccine alone (***Eimeria***) and group 3 birds treated with *Cp* alone. Two experimental groups were NE birds (birds exposed to a coinfection with *Eimeria* spp. vaccine and *Cp*): one of these groups, group 4 was not fed the probiotic (NE), whereas group 5 was fed the control diet supplemented with the probiotic (**NE-H57**). The last group (group 6) was not infected with *Cp* or with *Eimeria* spp. vaccine but fed the control diet supplemented with H57.

As described previously, the probiotic H57 (a bacterial spore-forming strain of *B. amyloliquefaciens*) was added to the basal diet from the first to the last day of the experiment (days 0–21), to one group of unchallenged chicks (group 6 or H57 birds), and one group of NE-

Table 1. Formulation and nutrient composition (g/kg) of the basal diet.

Ingredient	(g/kg)
Wheat	587.6
Soybean meal (46.5% CP)	316.0
Canola meal	20.0
Canola oil	30.0
Limestone	14.0
Sodium chloride	2.1
Sodium bicarbonate	3.5
Choline chloride	0.2
DL-Methionine	2.8
L-Lysine HCl	2.9
L-Threonine	3.7
Mono-dicalcium phosphate	14.7
Vitamin and mineral premix[1]	2.5
Calculated nutrient composition	
DM	900.5
CP	240.0
Crude fat	46.1
Crude fiber	34.3
ME (kcal/kg)	2,900
Calcium	9.0
Phosphorus	7.4
Available phosphorus	4.5
Sodium	2.0
Potassium	9.2
Chloride	2.3
Lysine	13.9
Methionine	5.9
Methionine + cystine	10.1
Threonine	12.2
Leucine	17.3
Isoleucine	10.0
Tryptophan	3.0
Arginine	15.2
Valine	10.9
Choline	16.4

[1]The premix supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 2,500 IU; vitamin E, 30 mg; vitamin K3, 2 mg; vitamin B1, 1.5 mg; vitamin B2, 8 mg; vitamin B6, 4 mg; vitamin B12, 0.02 mg; D-calcium pantothenate, 15 mg; folic acid, 2 mg; nicotinic acid, 45 mg; biotin, 0.135 mg; cobalt, 0.2 mg; copper, 6 mg; iron, 50 mg; iodine, 0.75 mg; manganese, 75 mg; molybdenum, 1 mg; selenium, 0.15 mg; Zn, 60 mg.

challenged chicks (group 5 or NE-H57 birds). The probiotic H57 was added to the basal diet (Table 1) at a concentration of 2×10^8 spores/g feed and was supplied as a freeze-dried preparation in a bentonite carrier. After feed mixing, the feed was stored at room temperature. Plating of serial dilutions of feed samples assessed the H57 populations in the diets. A content of H57 spores at an average dose 2.48×10^8 cfu/g feed was confirmed after microbiological analyses. The experimental diet was not medicated or supplemented with any antimicrobial growth promoters, coccidiostats, or feed enzymes.

Experimental Induction of Subclinical NE Disease Model

The most commonly used model for bacterial intestinal infections in broiler chicks is the NE challenge disease model (Prescott et al., 2016b). It can be developed in birds as a clinical or subclinical model; however, every protocol differs in the method of infection and outcome assessment. For the present study, a subclinical NE challenge disease model was used. A bird with subclinical NE does not display clinical signs of disease but still experiences infection. Our model was based on previous investigations and recommendations (Gholamiandehkordi et al., 2007; Shojadoost et al., 2013; Wilson et al., 2018), with modification, as the intention was to avoid severe clinical signs or mortality occurring in challenged birds. In the subclinical form of NE, clinical signs would appear mild or not appear; however, obvious NE disturbance/lesions in the small intestine should be found. Other investigators have achieved experimental NE in broiler chicks with *Eimeria* spp. and *Cp* through the administration of both pathogens by oral gavage. This method does not resemble the commercial environment and does not represent the natural process of infection. Most importantly, the oral gavage method introduces another predisposing factor to birds, that is handling stress.

In the current trial, both pathogens were delivered orally, *Eimeria* spp. in the drinking water and *Cp* in the feed. Briefly, the dual-infection system was used with a highly virulent *Cp* type A strain EHE-NE18 (CSIRO, Geelong, Australia), isolated in Australia from NE outbreaks and a commercially available anticoccidiosis vaccine containing 4 strains of *Eimeria* spp. (i.e., viable oocysts of *Eimeria acervulina, Eimeria*

Table 2. Experimental groups and treatment details for broiler chicks from days 0 to 21.

Groups and treatments	Day 0–8	Day 9	Days 10–14	Days 14 and 15	Days 16–20	Day 21
1. Control (basal diet)	All groups fed their diets. Feed intake and BW recorded on day 7. All groups tested for *Eimeria* spp. oocytes in excreta on day 8.	PBS[2]	All groups fed their diets. Feed intake and BW recorded on day 14.	Sterile broth[3]	All groups fed their diets. On day 20, all groups tested for *Eimeria* spp. oocytes in excreta.	Feed intake and BW recorded. One bird/replicate (6/treatment) was euthanized for necropsy and tissue sampling. All birds were euthanized for lesion scoring.
2. *Eimeria* (basal diet)		*Eimeria* vaccine		Sterile broth		
3. *Cp* (basal diet)		PBS		Broth inoculated with *Cp*[3]		
4. NE[1] (basal diet)		*Eimeria* vaccine		Broth inoculated with *Cp*		
5. NE-H57 (basal diet + H57)		*Eimeria* vaccine		Broth inoculated with *Cp*		
6. H57 (basal diet + H57)		PBS		Sterile broth		

Abbreviations: *Cp, Clostridium perfringens*; H57, *Bacillus amyloliquefaciens* strain H57; NE, necrotic enteritis.
[1]NE-infected birds were coinfected with *Eimeria* vaccine and *Clostridium perfringens*.
[2]PBS and the *Eimeria* vaccine were delivered in the drinking water (water was withheld for 3 h before treatment).
[3]Sterile broth and broth inoculated with *Cp* were mixed with feed (feed was withheld for 5 h before treatment).

maxima, Eimeria necatrix, and *Eimeria tenella*) suspended in PBS. To confirm *Eimeria*-free rooms, on day 0 (before bird arrival) and day 9 (before vaccine administration), swab samples from each cage (on day 0) or fresh excreta samples (on day 9) were collected and processed by a standard method for egg counts/g feces. To confirm *Eimeria* infection and oocyst shedding, the test with fresh excreta samples was repeated on day 14 and 21. The *Eimeria* oocyst and nematode egg counts are presented in supplementary material (Supplementary Table 1).

Eimeria spp. vaccine, containing live and attenuated oocytes at concentration 1.6 x 10^4 oocysts/mL, was used to induce moderate intestinal damage. To insure efficacy, a high dose of the vaccine (20x the manufacturer's recommended dose) was used in this trial. The vaccine was delivered in the drinking water to 9-day-old birds (Table 2). For the high dose of vaccine given to birds the oocyte content was calculated at approximately 8,000 oocytes/bird (or 1,000 oocytes/bird of *E. acervulina*, 2,000 oocytes/bird of *E. maxima,* 2,000 oocytes/bird of *E. necatrix*, and 3,000 oocytes/bird of *E. tenella*). The dose of 8,000 oocytes/bird was suspended in 0.5 mL PBS/bird. On day 9, all birds received water in a bell drinker and either treated with PBS (groups 1, 3, and 6) or vaccine (groups 2, 4, and 5). Before treatment exposure, water was withhold for 3 h. Care was taken that birds in each cage drank all the vaccine-treated water.

On day 14, birds were challenged with *Cp*, strain EHE-NE18, which is known to produce lesions typical of NE (Keyburn et al., 2010). The *Cp* isolate was incubated overnight (not shorter than 15 h or longer than 18 h) at 39°C in thioglycollate broth (**TGB**; Thermo Scientific, Australia), followed by overnight incubations in cooked meat broth (Oxoid, Australia), and then again in TGB to obtain the challenge inoculum. Birds infected with *Cp* (group 3) and birds infected with NE (groups 4 and 5) received freshly prepared broth culture containing *Cp* suspension (1.76×10^8 cfu/mL). Birds in 3 other groups (control, *Eimeria,* and H57) received the same amount of sterile TGB mixed in feed (Table 2). The *Cp* suspension was mixed manually with feed at a ratio of 1: 1.5 (vol/wt). Feed was withdrawn from all birds approximately 5 h before challenge. Chicks were fed the diet for 2 D. Unconsumed feed was weighed and disposed.

Assessment of Performance

Birds and feed consumed were weighed weekly (on days 0, 7, 14, and 21) and ADG, feed intake (**FI**), ADFI, and feed conversion ratio (**FCR**) were calculated. Performance parameters (BW, FI, and FCR) were adjusted for mortality. Mortality was recorded daily and reported as percentage (**%**) cumulative mortality (% dead birds vs. alive) at the end of the experiment. On day 21, all birds were euthanized by cervical dislocation and necropsied. BW of individual birds was measured before euthanasia to calculate liver weight (**LW**) relative to BW ratio (g LW/100 g BW).

Gross Pathology and Lesion Scoring

During the experiment, general health and welfare of birds were checked twice daily. Dead birds (and euthanized sick birds) were weighed and necropsied. After the treatment with vaccine and the treatment with *Cp*, birds were closely observed for morbidity or clinical signs of distress or disease. In this trial, few birds displayed mild or transitory signs of infection, such as ruffled feathers and depression (Figure 1B). Some birds also had signs of diarrhea, as indicated by vent condition or pasty vent (Figures 1C, 1D). The incidence of pasty vent was recorded by visual inspection based on the presence or absence of sticky feces in the vent area (De Cesare et al., 2017). This parameter can help with the assessment of NE as it reflects health of the GIT (i.e., presence or absence of diarrhea). Data on pasty vent were recorded on day 21 by 3 independent assessors, and the average of 3 measurements was used to calculate % of birds with pasty vent against birds with clean vent for each replicate.

The diagnosis of NE was performed using macroscopic and microscopic standards recommended by other investigators and reviewed by Smyth (2016). For a final diagnosis, data for vent condition, small intestinal lesion score, and histopathologic examination of the ileum (i.e., ileal mucosa integrity and enumeration of *Cp* bacteria attached to mucosa) were used. Gross pathology of intestinal mucosa was performed after the small intestine from each bird was incised longitudinally and digesta were removed. Immediately, tissue samples from the ileum of 2 birds/replicate (or 12/treatment) were collected for histology. Examination of the small intestine was performed by 2 individuals (who performed evaluation at the same time, with one of them being blind to the experimental treatments) and was based on a modified checklist presented in Table 3, using a 0–4 scoring system. Criteria for lesion scoring was based on previous recommendations (Prescott et al., 1978; Broussard et al., 1986; Gholamiandehkordi et al., 2007), with some adjustments to suit the mild form of subclinical NE anticipated in this trial (Table 3; Figures 2A, 2B). The intestine was scored for lesions at 3 sites: the duodenum, jejunum, and ileum, and the lesion scores were then recorded as the average across the 6 birds/replicate at each segment. The total lesion score was calculated as the sum of lesion scores for the 3 intestinal segments.

The intestinal content from the ileum of 12 birds/treatment was also collected in individual 50-mL conical Falcon centrifuge tubes, and the pH was determined with a conventional pH meter (MC80 model, TPS, Australia) immediately upon collection. Two measurements were taken, and the average was used for statistical analysis. Another small amount of ileal content was taken aseptically for enumeration of bacteria. Ileal content samples were serially diluted in PBS for enumeration of total aerobes, anaerobes, and coliforms, by conventional microbiological techniques (data not shown here). This test was not used for enumeration of

Figure 1. Birds displaying mild clinical signs of necrotic enteritis. Bird with clean vent (A) or pasty vent (B, C), both images taken on day 21; bird with depression/ruffled feathers (D), image taken on day 14, 6 h after feed mixed with *Cp*-inoculated broth was given to birds. Abbreviation: *Cp, Clostridium perfringens.*

Table 3. Diagnostic checklist for screening and scoring gut lesions for necrotic enteritis.[1]

Content/debris over mucosa[2]	Presence of gas mixed in content	Type of hyperemia or hemorrhage	Type of erosions/ ulcers	Type of necrosis	Number of events[3]	Summary of events & lesion scores[4]
Normal (brown/green, soft)	None	None, or few hyperemic areas (1–2)	None	None	0–1	Absent; 0
Watery, with white plaques	None or small amount	Some hyperemic areas (3–6)	Minor	Focal	2–3	Few present; 1
Greyish-creamy	Small amount	Scattered petechial or hyperemic areas (6–10)	Focal	Patchy (2-3 cm)	3–4	Some present; 2
Brown or bile-stained or fibronecrotic pseudomembrane	Moderate amount	Multiple petechial (>10) or hemorrhagic areas	Multifocal	Segmental (4–8 cm)	4–5	Typical of NE; 3
Blood in intestine or brown thick necrotic debris	Gas-filled (ballooned intestine)	Marked hemorrhage	Diffuse	Diffuse	4–5	Severe[5] lesions of NE; 4

[1]This diagnostic checklist is based on previous published work (Prescott et al., 1978; Broussard et al., 1986; Gholamiandehkordi et al., 2007) and the personal experience of the assessors. The checklist can be used for both forms of NE (subclinical and clinical). If familiar with gross examination of NE, use only last column on the right. If you are less experienced with NE diagnosis use 5 other columns on the left, and check for types of lesions found, then give the scores based on the number of events. For those involved in the control and treatment of NE farm level, as suggested by Smyth (2016), it is essential to use a checklist in association with microbiological testing (to confirm presence of *Clostridium perfringens*) and histopathological examination (to confirm the presence of lesions, oocytes and gram positive rods) of the sampled parts of small intestine.

[2]If present.

[3]Any of the observations recorded in the 5 columns on the left.

[4]Scoring system used 0–4.

[5]Birds could die at this stage.

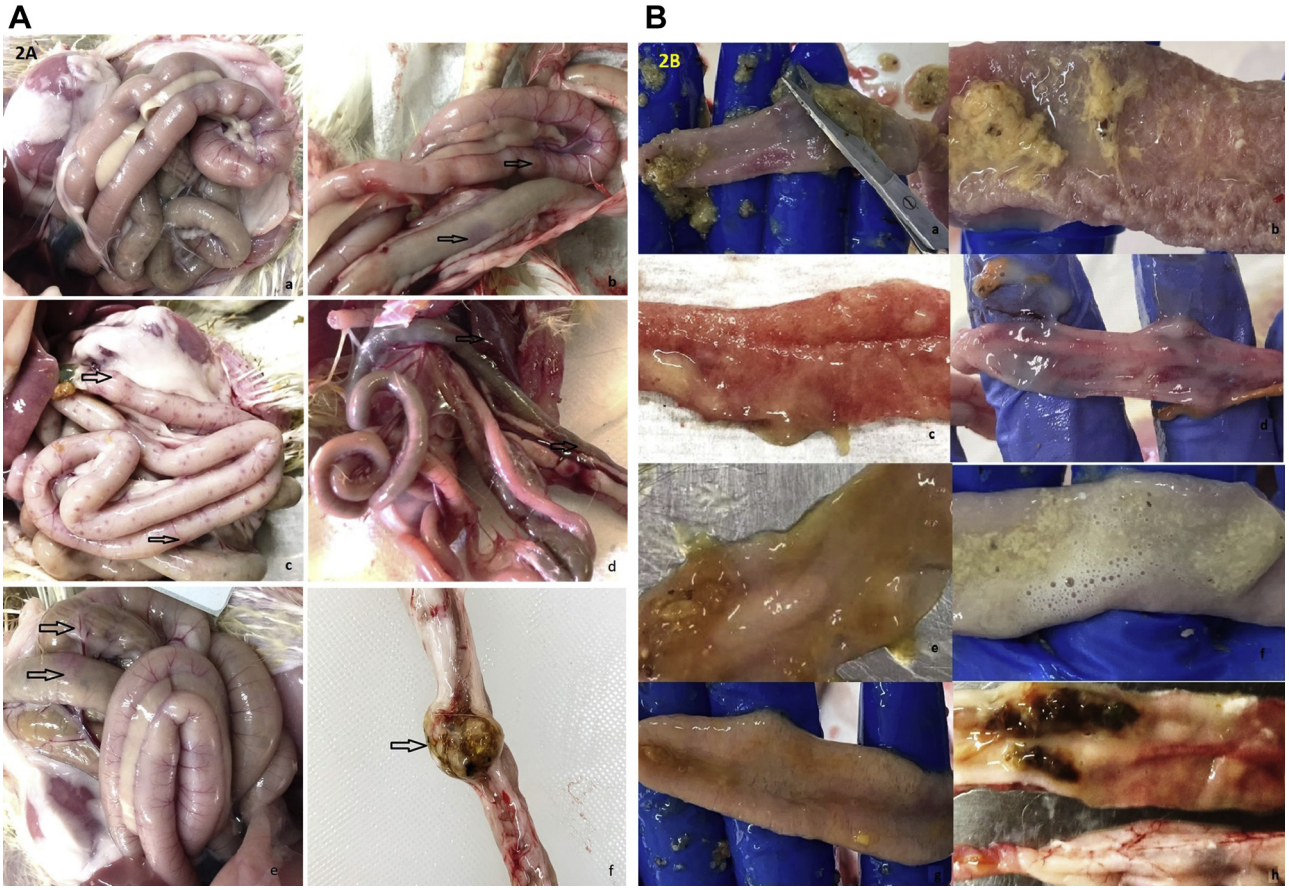

Figure 2. Gross pathology of the ileum from broiler chicks showing some of the criteria used for lesion scoring. (A) Images show the outside appearance of the ileum in the abdominal cavity of a control chicken (a), a chicken demonstrating ileal focal bruising (b), scattered petechial areas of ileum (c), multifocal bruising and part of the ileum filled with blood and a dark liver (d); image with thin walls and watery gut contents (e), and a ballooned intestine (e, f). (B) Images show the inside appearance of ileal mucosa of a control chicken with a hyperemic area representing Payer patches (a); a watery content with white plaques (b); a creamy content covering hyperemic mucosa (c, d); focal necrosis with bile-mixed content (e); and a watery content with moderate amount of gas (f); mucosa covered with necrotic pseudomembrane or a "Turkish towel" appearance (g); and numerous necrotic foci with brown content (h).

Cp specifically, as there have been reported failures to culture ileal contents for the bacteria of interest (Smyth, 2016). Therefore, for *Cp* enumeration, as suggested by Park et al. (2008), the Brown and Hopps's Gram staining method was used (Brown and Hopps, 1973).

Histological Evaluation

Intestinal segments from the ileum (proximal to the ileocecal junction) were fixed in 10% phosphate-buffered formalin. Thereafter, sample tissues were dehydrated, cleared, and embedded in paraffin wax. Serial sections of 5 µm were cut, and 4 consecutive sections of each paraffin-embedded segment were deparaffinized in xylene, rehydrated, and stained with respective stains, that is hematoxylin and eosin (**H&E**), periodic acid–Schiff, and Gram stain (Figure 3). Stained slides were scanned using an Aperio ScanScope slide scanner at 40x magnification, and digital image files were created. Images were viewed and observations were annotated and measured using Aperio ImageScope 12.3.0.5056 slide-viewing software. The H&E-stained slides from ileum samples (Figure 3A) were used to run mucosal measurements by 2 independent investigators. Aperio tools were used to run morphometry measurements of 6 well-oriented crypt–villus units/bird (in total 72 villi/treatment). The criterion for villus selection was based on the presence of intact lamina propria, orientation (vertical), and villus integrity in general. Villus height (µm) was measured from the tip of the villus to the villus–crypt junction, whereas crypt depth (µm) was defined as the depth of the invagination between adjacent villi, and then, the villus height:crypt depth (**VH:CD**) ratio was calculated.

The Gram stain was used to detect *Cp* bacteria close or attached to the ileal mucosa (Figure 3B). Individual bacteria are not easily detected with H&E; the Gram stain is a reliable for the detection of both gram-positive and gram-negative bacteria and also provides an excellent contrast between bacteria and nonviable debris (Brown and Hopps, 1973). For *Cp* counts, screenshots of the Aperio ImageScope slides at 40x were taken from each tissue section (5 images from each bird or 60/treatment) and gram-positive bacteria counted and expressed as average/image. Gram-positive bacteria

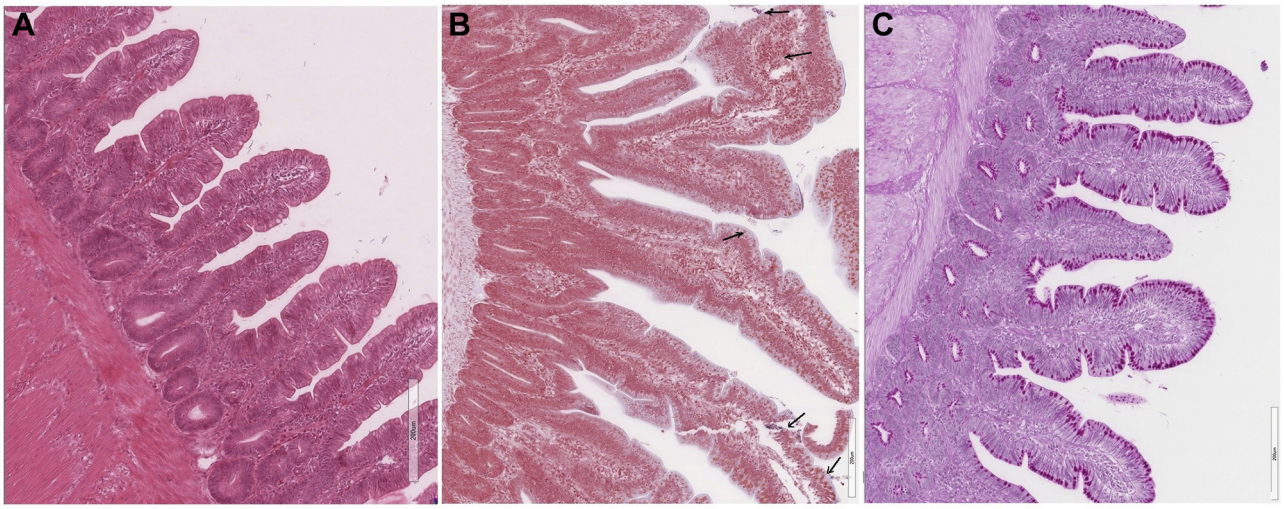

Figure 3. Examples of staining of ileal tissue sections with hematoxylin & eosin (H&E), Gram stain, and Periodic Acid–Schiff (PAS) that were used to run morphometric measurements, detect and count gram-positive bacteria, and count goblet cells. Images stained with H&E showing normal villi (A), stained with Gram showing damage of epithelium of villi by *Eimeria* and attachment of *Clostridium perfringens* on mucosa (B), and stained with PAS showing goblet cells and presence of mucus in the crypts (C).

were stained dark blue by light microscopy. Gram reaction, microscopic morphology (shape and size of bacteria), and attachment or proximity to the mucosa were used as a criteria to differentiate *Cp* from other gram-positive bacteria. *Eimeria* oocytes and infiltrated white blood cells can also be seen better with this stain (Figure 3B). Periodic acid-Schiff staining was used to stain mucus droplets in goblet cells (**GC**) and brush border of the absorptive epithelium (Figure 3C). For GC counting, 2 villi per sample (24/treatment) were used, and the GC density was expressed as the number of cells/100 μm villus height. Intraepithelial immune cells (**IEIC**) represented mainly by intraepithelial lymphocytes and heterophil leukocytes were differentiated as nonepithelial cells found above the epithelial basal lamina and between epithelial cells. The IEIC were counted on Gram-stained slides, using the same villi as GC, and expressed as the number of cells/100 μm villus height. Thereafter, the same villi were used to calculate villus absorptive surface area, based on height and average width of each villus, using the formula by Sakamoto et al. (2000):

Villus absorptive surface area = $2\pi \times$ (average villus width/2) $\times$ villus height.

The average villus width was found from width measurements at the top, middle, and base of the villus.

Statistical Analysis

Mean ± SD for each treatment were calculated and differences between treatments were analyzed using MiniTab 17 software. A 1-way ANOVA procedure was performed with treatment as a single factor, and Tukey's multiple comparison test was used to determine significant differences among treatments. For performance parameters, each cage was considered as an experimental unit (replicate), whereas for the remaining parameters, analysis was based on randomly selected birds from all replicates of each group. Data on mortality were calculated as percentages of dead birds vs. live birds. There was a large variance in the percentage mortality data distribution, that is 1 or 2 deaths per replicate were converted as 12.5 or 25 vs. 0% deaths per replicate. To address this, a logarithmic transformation (log10) was applied. Differences were considered significant at $P < 0.05$.

RESULTS

Throughout the 3-wk study, birds generally appeared clinically normal, although after the establishment of NE on day 14, some birds in group 4 did exhibit mild clinical signs of NE (Figure 1).

Bird Performance and Gut Health

Data on bird performance and gut health indices are presented in Table 4. Bird performance data are presented only for the last week (day 14–21), after induction of NE, and for the total experimental period (day 0–21). There were no significant differences between treatments for BW of birds on days 0, 7, and 14 (Supplementary Table 2). On day 21, birds fed H57 were significantly heavier than NE birds (859 g vs. 806 g; $P = 0.006$) but not control birds (Supplementary Table 2). Interestingly, NE-H57 chickens achieved a BW similar to control birds and higher than NE birds (845 g vs. 806 g; $P = 0.031$). There were no significant differences in ADG of birds between days 14 and 21 (Table 4). During week 33, NE-H57 birds performed better than NE birds (59.5 g/day per bird vs. 55.4 g/day per bird; $P = 0.054$). The ADG for the entire growing period (day 0–21) revealed significant differences between H57 and NE birds (39.2 vs. 36.6 g/day per bird; $P < 0.005$). Birds in the NE-H57 group also grew faster than NE-infected

Table 4. Performance and intestinal health parameters of broiler chicks for all treatments.

Parameters	1. Control	2. *Eimeria*	3. *Cp*	4. NE	5. NE-H57	6. H57	Pooled SD	*P* value[1]
Weight gain (g/bird/day)								
Day 14–21	60.7 ± 4.4	58.8 ± 2.4	56.5 ± 3.5	55.4 ± 5.1	59.5 ± 3.6	61.8 ± 3.3	3.80	0.054
Day 0–21	38.5 ± 0.9^{a,b}	37.8 ± 0.7^{a,b}	37.3 ± 1.4^{a,b}	36.6 ± 1.6^{b}	38.5 ± 0.9^{a,b}	39.2 ± 1.1^{a}	1.12	0.005
Feed intake (g/bird/day)								
Day 14–21	98.8 ± 3.1	98.1 ± 4.8	98.9 ± 4.9	98.8 ± 3.8	96.9 ± 3.5	96.3 ± 4.3	4.10	0.811
Day 0–21	49.1 ± 1.7	49.4 ± 2.5	49.8 ± 2.0	49.8 ± 4.0	49.4 ± 1.9	48.7 ± 2.0	1.93	0.923
Feed conversion ratio (g feed/g gain)								
Day 14–21	1.63 ± 0.13^{a,b}	1.67 ± 0.07^{a,b}	1.76 ± 0.13^{a}	1.79 ± 0.12^{a}	1.63 ± 0.11^{a,b}	1.56 ± 0.04^{b}	0.09	0.006
Day 0–21	1.27 ± 0.03^{b,c}	1.31 ± 0.04^{a,b,c}	1.34 ± 0.06^{a,b}	1.36 ± 0.03^{a}	1.28 ± 0.03^{b,c}	1.24 ± 0.03^{c}	0.04	0.001
Mortality (log)								
Cumulative	2.27 ± 0.40	1.67 ± 0.80	1.67 ± 0.80	1.50 ± 0.55	1.67 ± 0.52	1.17 ± 0.41	0.61	0.168
Pasty vent (%)								
Day 21	17.6 ± 9.70^{b}	26.8 ± 7.84^{a,b}	29.5 ± 11.92^{a,b}	41.4 ± 12.73^{a}	19.1 ± 4.78^{b}	17.1 ± 7.04^{b}	9.42	0.001
Lesion score[2]								
Day 21	1.17 ± 0.82^{c,d}	2.56 ± 0.40^{b}	2.78 ± 0.54^{b}	5.67 ± 0.30^{a}	2.06 ± 0.65^{b,c}	0.83 ± 0.35^{d}	0.18	0.001
Liver weight (g/100 g BW)								
Day 21	2.78 ± 0.20^{b}	2.89 ± 0.24^{a,b}	2.76 ± 0.18^{b}	2.99 ± 0.24^{a,b}	2.92 ± 0.20^{a,b}	3.24 ± 0.20^{a}	0.21	0.006
pH (ileum)								
Day 21	7.74 ± 0.37^{a}	7.76 ± 0.52^{a}	7.82 ± 0.72^{a}	7.62 ± 0.70^{a}	6.56 ± 0.25^{b}	7.08 ± 0.36^{a,b}	0.52	0.001

Abbreviations: *Cp, Clostridium perfringens*; H57, *Bacillus amyloliquefaciens* strain H57; NE, necrotic enteritis.
[1]Values in the same row with different superscripts are significantly different.
[2]Lesion scores are an average for all 3 segments of the small intestine: duodenum, jejunum, and ileum.

birds and at a similar rate to control birds (38.5 vs. 36.6 vs. 38.5 g/day per bird). Relative to BW, LW of H57 birds were significantly greater ($P < 0.05$) than for control and *Cp* birds.

There were no significant treatment effects on FI of broiler chicks challenged with pathogens or diets supplemented with H57 (Table 4). However, there were significant differences in FCR ($P < 0.006$) among treatments at the end of week 3. Despite consuming similar amounts of feed, NE birds gained significantly less BW than H57 birds. The greatest difference in FCR was between NE-H57 and NE birds (1.28 vs. 1.36; or a decrease in FCR of 0.08 units; $P < 0.001$). The NE-H57 birds performed similarly to control birds (FCR = 1.27; Table 4), whereas H57 birds had a small decrease of 0.03 units in FCR when compared with control birds (1.24 vs. 1.27; $P = 0.051$).

Mortality was very low. There were no significant differences between treatments in cumulative mortality (from day 0 to day 21; Table 4), and no deaths were due to NE. The NE birds had a higher occurrence of diarrhea (pasty vent) than birds infected with either *Eimeria* alone or *Cp* alone (41 vs. 27 and 29%, respectively; $P < 0.001$; Table 4). Birds in the NE-H57 group had a lower incidence of pasty vent than NE birds, but similar to control and H57 birds (19 vs. 18 and 17%, respectively). Pasty vent and lesion scores were correlated (Pearson's r = 0.56; $P < 0.001$).

The intestinal gross pathology conducted on day 21 revealed obvious mucosal damage measured by lesion score of the small intestine (Table 4), with NE birds having a significantly ($P < 0.001$) higher lesion score than all other groups. Most of the outer intestinal surface had focal or multifocal bruising, scattered petechial areas, and part of the ileum was filled with gas or watery contents (Figure 2A). Typically, the inner intestinal surface of these birds displayed moderate mucosal lesions (Figure 2B) with some birds revealing severe necrotic lesions. On day 21, when compared with NE birds, the NE-H57 birds had fewer lesions (5.67 vs. 2.10; $P < 0.05$). Birds infected with *Eimeria*-alone or *Cp*-alone also had fewer lesions than NE birds (2.78 and 2.56 vs. 5.67; $P < 0.001$). In *Eimeria* birds, lesions were typically coccidia infection, with microscopic examination of the ileum demonstrating oocysts and macrogametocytes in the epithelium and lumen (Figures 4A, 4B). Excreta examination also showed significantly more *Eimeria* oocytes in birds treated with the *Eimeria* vaccine (see Supplementary Table 1). Gram-stained slide counts confirmed the presence of gram-positive *Cp* and *Eimeria* oocytes in NE birds (Figures 3B and 4A–4D). On day 21, the number of bacteria counted in the ileum mucosa of NE birds was significantly higher ($P < 0.001$; Figure 5) when compared with birds infected with *Cp* alone. However, birds in the NE-H57 group had a significantly lower ($P < 0.001$) number of *Cp* bacteria/image than NE birds. The 3 other groups had similar numbers of *Cp* bacteria on the mucosa, which was significantly lower ($P < 0.001$) than the NE-infected, *Cp*-alone, and NE-H57 groups. Interestingly, the dietary addition of H57 decreased the pH of ileum digesta in NE-H57 birds that was approximately 10 times lower ($P < 0.001$) than in NE birds (6.56 vs. 7.62; $P < 0.001$), and all other groups receiving the basal diet (Table 4).

Histopathology and Morphometric Evaluation

Macroscopic lesions were typically found on the jejunal and ileal mucosal surface, with lesions extending into the duodenum in some birds. Microscopic evaluation of ileal lesions is the only one reported here. Only

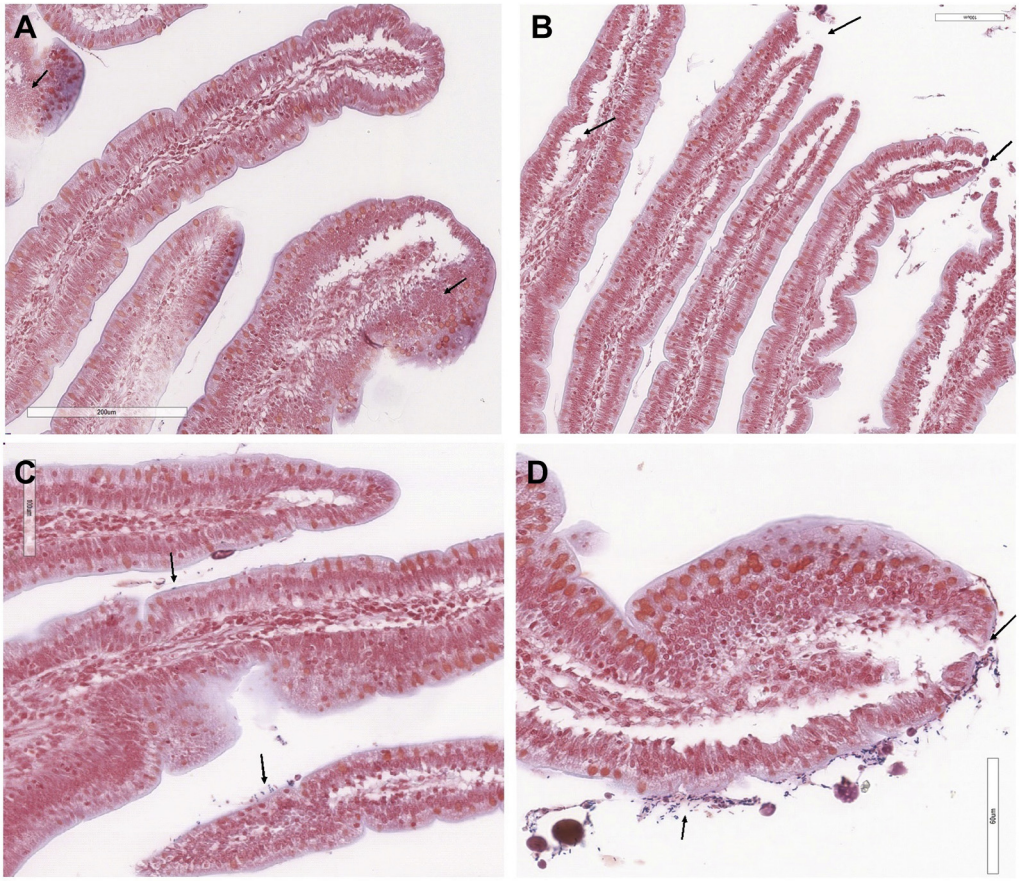

Figure 4. Histopathology of villi after infection with *Eimeria* spp. alone (A, B) and coinfection with *Eimeria* spp. and *Cp* (C, D) from samples collected on day 21 in a subclinical necrotic enteritis model. Arrows indicating *Eimeria* spp, *Cp*, mucus presence, and epithelium damage. Abbreviation: *Cp, Clostridium perfringens*.

a few samples were cut from areas with coagulative lesions. In some cases, the fibrinonecrotic material had been shed from the surface (or removed with the gut contents), and only a relatively smooth remaining surface was sampled. Figures 6A–6F shows ileal villi images from the 6 treatments. Characteristic microscopic lesions of NE (mucosal damage and necrosis) were present in almost all samples from NE birds and in some NE-H57 birds. Lesions were either superficial (at the villus tip) or deeper, with damage to a single villus or group of villi (Figure 6D), and were accompanied by inflammatory changes, such as congestion of the lamina propria and infiltration with fibrin and immune cells (mononuclear cells and heterophils). In severe cases, owing to coagulative necrosis, the entire epithelium was separated, exposing the underlying lamina propria, which was infiltrated by inflammatory cells (Figure 7A). Some inflammation and epithelial damage was also present in *Eimeria* birds as it is a characteristic of coccidiosis; however, detachment or erosion of epithelium was not seen (Figure 4A). Microscopic examination revealed *Eimeria* oocysts (a mixture of small- and medium-sized oocytes) invading the epithelium and, in some cases, the lamina propria (Figure 4D). In NE birds, Gram staining revealed a variable number of gram-positive, long, and rod-shaped bacteria, attached to the epithelium and/or invading the lamina propria and crypts (Figures 7A–7C). Debris containing clumps of long rod-shaped bacteria and sloughed degenerated cells were also noted in the lumen. Villi from samples of NE-H57 birds were frequently nearly normal in appearance, with cloudy swelling of epithelium in some areas and mild infiltration of immune cells but without damage or profuse infiltration with *Eimeria* or *Cp* (Figure 6E). The level of damage in all treatments was consistent with lesion scores.

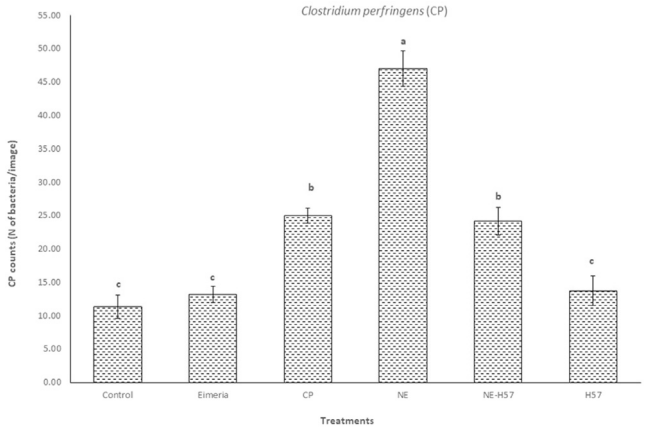

Figure 5. Ileal *Clostridium perfringens* (*Cp*) bacteria counts (number of bacteria/image) from Gram-stained histologic sections. Data are expressed as mean ± SD (n = 60). Different letters in each bar indicate statistical differences between treatments ($P < 0.05$).

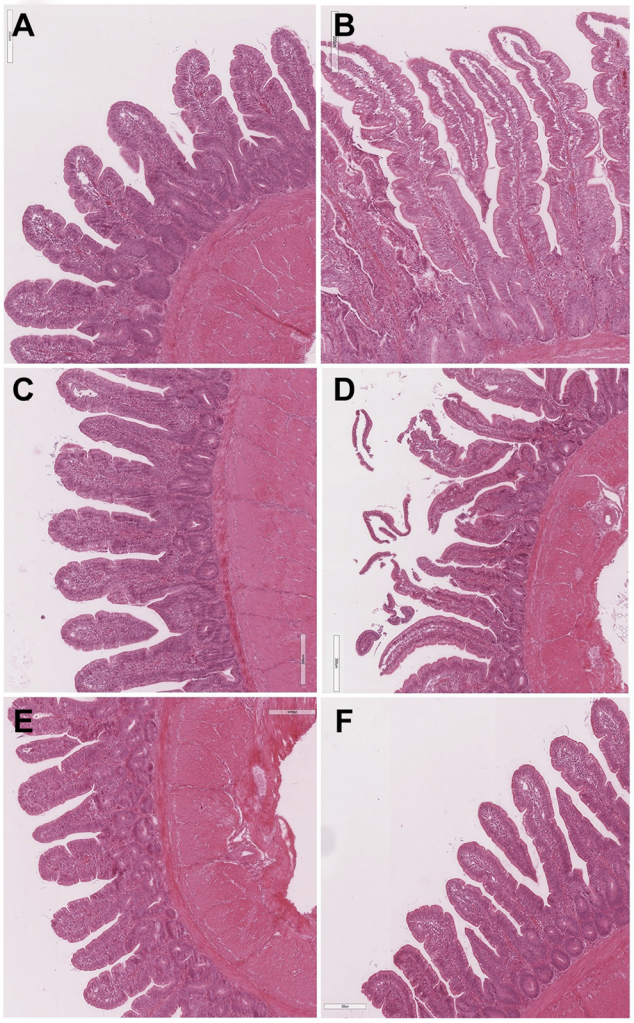

Figure 6. Histologic section (H&E) of ileum from birds of all treatments. Images show intestinal wall integrity from a control bird (A); from an *Eimeria*-infected bird (B); from a *Cp*-infected bird (C); from a NE-infected bird, showing villus damage and congestion of mucosa (D); from a NE-H57-infected bird (E); and from a H57-tretaed bird (F). Abbreviations: *Cp, Clostridium perfringens*; H&E, hematoxylin & eosin; H57, *Bacillus amyloliquefaciens* strain H57; NE, necrotic enteritis.

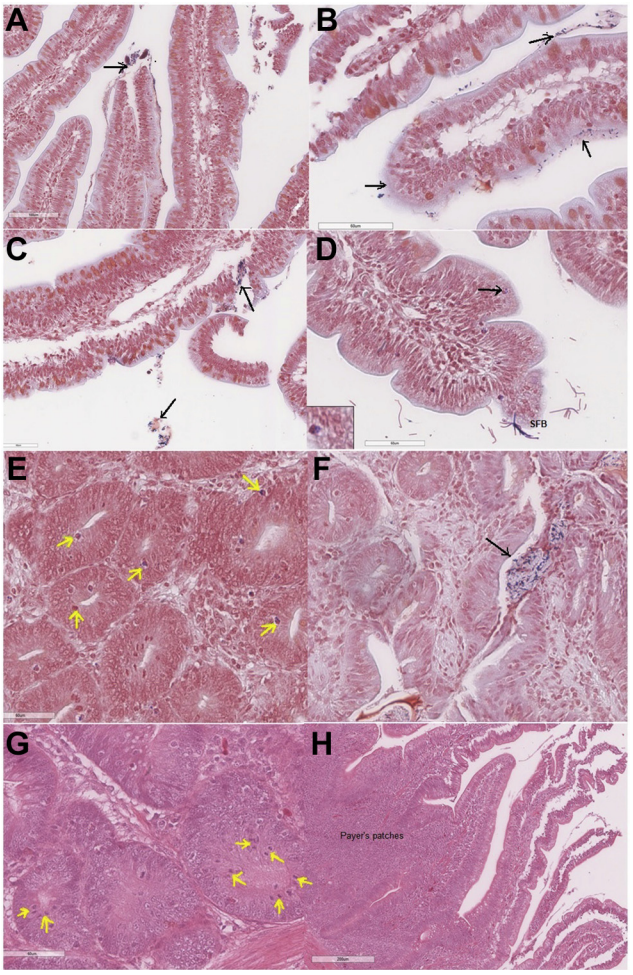

Figure 7. Histopathology of ileum from chickens infected with necrotic enteritis. H&E and Gram staining images that confirm the pathogenesis of NE; *Eimeria* infiltrates the apical villus and stimulates mucus production, preparing the environment for the *Cp* to penetrate and colonize (black arrows) (A, B); damage of tip of villus, exposure of lamina propria, and growth of *Cp* in NE birds (C); villus infiltration with intraepithelial immune cells and segmented filamentous bacteria (SFB) in NE-H57 birds (D); crypt infiltration with immune cells (yellow arrows) in NE-H57 birds (E); colonization of *Cp* deeper into in the crypts in NE birds (F); hyperplasia of cryptal epithelium associated with mitotic figures (yellow arrows) (G); and Peyer's patches in the crypt of Lieberkühn zone (H). Abbreviations: *Cp, Clostridium perfringens*; H&E, hematoxylin & eosin; H57, *Bacillus amyloliquefaciens* strain H57; NE, necrotic enteritis.

Table 5 presents data on ileal mucosal morphometry measurements including villus height, crypt depth, and villus absorptive area. Goblet cells and IIC numbers are also presented; Both, exposure to pathogens and dietary supplementation with H57 affected villus height in all birds. On day 21, there was a significant increase ($P < 0.001$) in villus height of birds exposed to *Eimeria* (676 µm), *Cp* (495 µm), NE (467 µm), NE-H57 (443 µm), and H57 (421 µm) when compared with that of control birds (397 µm). The villi from *Eimeria* birds were significantly taller and thinner (data for thickness not shown) than in all other treatments. Crypt depth was less affected. There was a significant difference between *Eimeria* birds and all other treatments (Table 5), with these birds having a deeper crypt (231 µm; $P < 0.001$); the VH:CD ratio was consistent with villus height data, with *Eimeria* birds having the highest ratio and control birds the lowest (2.95 vs. 1.96; $P < 0.001$). The effect of treatment was less noticeable on total villus absorptive surface area. Birds in the *Eimeria* group had greater surface area than control birds (surface area, 0.23 vs. 0.19 mm^2; $P = 0.015$).

On day 21 (or 12 D after *Eimeria* administration), GC numbers in the ileum of all birds infected with *Eimeria* (i.e., *Eimeria*, NE, and NE-H57 birds) were significantly decreased compared with control and H57 birds (Table 5). Birds in the *Eimeria* group had the lowest number of GC (18.0/100 µm villus height), and birds in the H57 group had the highest number of GC (24.2/100 µm villus height). The number of IEIC was significantly higher in NE-H57 birds (6.8/100 µm villus height; $P < 0.001$) than in all other treatments, most probably

Table 5. Ileal villi morphometric parameters, GC, and IEIC in broiler chicks.

Parameters	1. Control	2. *Eimeria*	3. *Cp*	4. NE	5. NE-H57	6. H57	Pooled StDev	*P* value[1]
Villus height (µm)	397 ± 77.9^{e}	676 ± 134.2^{a}	495 ± 89.2^{b}	467 ± 74.7^{c}	443 ± 45.9^{c,d}	421 ± 46.5^{d,e}	83.54	0.001
Crypt depth (µm)	211 ± 54.7^{b}	231 ± 35.6^{a}	206 ± 26.2^{b}	203 ± 33.1^{b,c}	204 ± 22.8^{b}	192 ± 21.7^{c}	34.22	0.001
Villus/Crypt ratio	1.96 ± 0.48^{e}	2.95 ± 0.50^{a}	2.43 ± 0.47^{b}	2.33 ± 0.35^{b,c}	2.19 ± 0.27^{c,d}	2.21 ± 0.28^{d}	0.40	0.001
GC counts/100 µm villus height	23.8 ± 3.14^{a,b}	18.0 ± 4.06^{d}	22.2 ± 4.10^{a,b,c}	20.6 ± 3.28^{c,d}	21.2 ± 2.85^{b,c}	24.2 ± 3.38^{a}	3.50	0.001
IEIC/100 µm villus height	3.04 ± 1.06^{c}	4.82 ± 0.56^{b}	4.83 ± 0.39^{b}	4.70 ± 1.14^{b}	6.52 ± 0.87^{a}	4.44 ± 0.54^{b}	0.81	0.001
Absorptive surface (mm^2)	0.19 ± 0.05^{b}	0.23 ± 0.06^{a}	0.20 ± 0.04^{a,b}	0.20 ± 0.05^{a,b}	0.20 ± 0.03^{a,b}	0.20 ± 0.04^{a,b}	0.05	0.015

Abbreviations: *Cp*, *Clostridium perfringens;* GC, goblet cell; H57, *Bacillus amyloliquefaciens* strain H57; IEIC, intraepithelial immune cells (lymphocytes and heterophils); NE, necrotic enteritis.
[1]Values in the same row with different superscripts are significantly different.

because of an increase of infiltration of lymphocytes and heterophils to the epithelium (Figure 7E). Control group birds had the lowest number of IEIC (3.0/100 µm villus height).

DISCUSSION

A subclinical NE challenge model was developed to assess effects of H57 on gut health and performance of broiler chicks. The model successfully induced subclinical NE without excessive bird handling and the additional stress associated with gavage. The model also demonstrated the utility of pasty vent as a noninvasive indicator of gut health. Gut evaluation was focused on the small intestine, as this is primarily where digestion and absorption take place. In particular, the ileum was chosen as the organ of interest as it is the distal section of the small intestine and also has involvement in mucosal immunity. The ileum mucosa contains specialized immune cells, including microfold cells, Paneth cells, intraepithelial lymphocytes, and organized lymphoid structures such as Peyer's patches, which play important roles in the regulation and stimulation of gut immunity (Allaire et al., 2018). Conditions in the ileum are more favorable for microbial growth (including growth of probiotic bacteria) compared with the more proximal small intestine. The pH (close to neutral) and a longer transit time through the ileum create a dynamic environment for microbial metabolism (Booijink et al., 2010; Gerritsen et al., 2011).

Coinfection With Eimeria *and a Pathogenic Cp-Induced Subclinical NE*

Subclinical NE was successfully induced in birds coinfected with a high dose of *E.* spp. vaccine and a pathogenic strain of *Cp* bacteria. The NE disease induced in birds in this experiment was subclinical, with few clinical signs of infection. Mortality was low in all treatments, and based on necropsy, no mortalities were due to NE infection. Within h of post-*Cp* challenge, some birds in the coinfected group (i.e., NE-infected birds) displayed mild signs of NE (Figure 1), which resolved completely within 24 h. Infection with NE caused a slight decrease in bird performance. Although, all birds appeared healthy before euthanasia, at gross examination, some birds displayed mucosal inflammation and injury. Birds infected with both pathogens were diagnosed with subclinical NE, as they developed NE lesions on the intestinal wall, had histopathologic alterations of mucosal structure, and demonstrated elevated numbers of *Cp* bacteria in ileal tissue. Small intestinal lesions in NE birds were significantly increased ($P < 0.001$) when compared with those in H57, *Eimeria*, *Cp*, or NE-H57 birds. Birds treated with either *Eimeria* or CP displayed less severe intestinal damage and lower lesion scores than NE birds, confirming the absence of NE in these birds. Administration of *Cp* culture caused a slight inflammatory response (as indicated by a small increase in lesion scores and IEIC, when compared with control birds);

however, these changes were significantly lower than those in NE-infected birds (2.78 vs. 5.67) and comparable with those in *Eimeria*-infected birds (2.56). Lesions in *Cp* birds were in the form of hyperemic patches or scattered petechial areas that is more likely to be signs of a slight inflammation and dysbacteriosis (Teirlynck et al., 2011). A small disturbance in the ADG and FCR of these birds was also found but less than those of NE birds and comparable with those of *Eimeria* birds.

Histopathology of the ileal mucosa in NE birds demonstrated significant villus morphologic alterations, including focal erosions of epithelial cells, exposure of the lamina propria, and fusion of adjacent villi (Figures 6D and 7A, 7C). As suggested by Witlock and Ruff (1977), oocyst formation underneath the epithelium causes its detachment in sheets. Damage of the ileal epithelium and mucus production by *Eimeria* appears to facilitate dissemination and colonization of *Cp* deeper into the mucosa (Collier et al., 2008; McGuckin et al., 2011; Ficko-Blean et al., 2012), extending in some occasions to crypts (Figures 7C, 7F) and causing focal necrosis. The growth and colonization of the mucolytic *Cp* on ileal tissue after administration with a high dose of *Eimeria* vaccine caused intestinal lesions in the present study. Moreover, the diet used in this study (wheat–soybean meal diet) could have also predisposed to *Cp* colonization and infection (Annett et al., 2002; Jia et al., 2009).

Gram-stained images from the ileum (Figures 4B, 4C and 7A–7C) supported the proposed pathogenesis for NE (Timbermont et al., 2011; Prescott et al., 2016a), demonstrating that enterocyte damage caused by *Eimeria* enabled *Cp* access to binding sites in the mucosa and villus damage and necrosis. This did not happen in birds infected with either *Eimeria* or *Cp*. Essential to NE pathogenesis is a large number of *Cp* lining the epithelium and colonizing crypts (Figure 7D), with a further spread to the submucosa after necrosis and sloughing of the mucosa (Prescott et al., 2016a). The number of *Cp* bacteria counted in ileal tissue slides of the NE birds was greater than in either *Eimeria*-alone, *Cp*-alone, or NE-H57 birds (Figure 5). *C. perfringens*is a gram-positive, spore-forming anaerobic bacterium, which is ubiquitous in nature, and found in all poultry environments, including intestinal contents of healthy birds (Dahiya et al., 2005). Its presence is not necessarily associated with enteric infections or NE (Diaz et al., 2016), as long as the mucosal barrier is intact. *C. perfringens* bacteria were present in both the lumen and attached to the mucosa. The presence of *Cp*, intimately associated with necrotic lesions of intestinal mucosa, is essential for the diagnosis of NE. Hence, Gram staining of ileal tissue is a more accurate and reliable method for the diagnosis of NE than *Cp* counts from ileal digesta (Woods and Walker, 1996; Park et al., 2008).

Morphometry and Integrity of Ileal Mucosa

Villus height was increased in all treated birds but controls. Increased villus size is associated with activated cell proliferation (Yasar and Forbes, 1999). In normal circumstances, the intestinal epithelial cells undergo continual renewal, and cell loss at the villus tip is compensated by stem cell mitosis within the crypts of Lieberkühn (Williams et al., 2015). In chickens, normal cell proliferation is confined mainly in the crypts but can also occur along the villus (Geyra et al., 2001). In this study, a detailed observation of the crypts revealed more mitotic figures in the crypt epithelium from treated birds than in controls (Figure 7G); however, this was not quantified for all treatments. It has been shown that crypt cell hyperplasia occurs as a secondary response to inflammation or epithelial damage (coccidiosis and NE in our study), but it can also occur in the absence of any damage and is influenced by certain nutrients and immune signals (Ray and Johnson, 2014). Villus lengthening and the VH:CD ratio (but not absorptive area) was increased in both H57-fed groups (H57 and NE-H57 birds; Table 4), when compared with controls, demonstrating increased proliferation in these birds. In the NE-H57 birds, pasty vent occurrence decreased, also indicating preserved ileal barrier function.

Villi from *Eimeria*-infected birds were significantly longer and thinner than in other treatments (Figures 4 and 6B; Table 5). Previous investigators have suggested that villus atrophy and fusion result from *Eimeria* spp. infections 4 to 5 D after exposure (Witlock and Ruff, 1977; Williams, 2005; Sharma et al., 2015). In the present study, longer villi were found in *Eimeria* birds on day 11 after vaccine administration. Regenerative changes (such as lengthening of villi and hyperplasia of cryptal epithelium) may have occurred during the recovery phase of the disease to compensate disturbed absorption. The absorptive surface was also significantly increased in *Eimeria*-infected birds when compared with control birds (0.23 mm^2 vs. 0.19 mm^2).

Changes in absorption occurring in coccidiosis and NE have been linked to changes in intestinal permeability, a feature of intestinal barrier function (Williams, 2005; Ducatelle et al., 2018). Many luminal and systemic factors can independently influence barrier function and cause leakage of plasma proteins and watery diarrhea (Quigley, 2016; Camilleri, 2019). In coccidiosis and NE, intercellular connections within the epithelium are disrupted (due to erosions and ulcerations caused by *Eimeria* oocytes and *Cp*), and barrier dysfunction contributes to diarrhea via a leak-flux mechanism. These observations were confirmed by electron microscopy of the ileal mucosa of broilers exposed to NE and H57, which revealed significant villus damage, including enterocyte injury, and loss of cellular integrity in NE birds, when compared with other treatments (Shini et al., 2019). Ultrastructure of apical junctional complexes was also altered, potentially resulting in barrier loss and mucosal injury (Vogelmann et al., 2004). Coccidiosis and NE-related diarrhea and malabsorption have been associated with reduced nutrient digestion and performance (Turk, 1972; Sharma and Fernando, 1975; Witlock and Ruff, 1977). However, epithelial damage in *Eimeria* birds appeared less severe than in NE birds, therefore a higher

absorptive area was available in these birds, reflecting their slightly greater ADG than NE birds.

The highest number of GC was found in H57 birds (24.23/μm villus height) suggesting the presence of a more intact/robust mucus layer protecting the intestinal epithelium. Fewer GC were found in NE (20.63/μm villus height) and *Eimeria* birds when compared with control birds (18.03 vs. 23.8/100 μm villus height). This was expected for both treatments. While overall villus height increases, GC numbers remains low, even though normal turnover between all intestinal epithelial cells is 3 to 7 D (Umar, 2010; Birchenough et al., 2015), indicating a slower turnover of GC in the damaged epithelium of ileal villi. Mucus secreted by ileal epithelial cells onto their surface represents a major protection mechanism against bacteria. However, small intestinal mucus is a critical source of nutrients for *Cp* as it allows these bacteria to form localized microcolonies on the mucosal surface (McGuckin et al., 2011; Ficko-Blean et al., 2012).

Efficacy of H57 on Gut Health and Performance of Broiler Chicks

There have been mixed reports on the effects of probiotics on performance indices when fed to broiler chicks. Some investigators have reported positive effects on bird performance (Patel et al., 2015; Palamidi et al., 2016; De Cesare et al., 2017; Pereira et al., 2019), whereas others have not seen any significant effect of specific probiotics (Olnood et al., 2015; De Souza et al., 2018; Zarei et al., 2018; Araujo et al., 2019). The addition of H57 to the diet of challenged birds (NE-H57 birds) from day 0 to day 21 ameliorated the damaging effects of NE on gut mucosa. Potentially, ileal colonization with the probiotic H57 prevented growth of *Cp* on the mucosa. Fewer *Cp* were found in NE-H57 birds when compared with NE birds. This study revealed that cellular architecture of the intestinal epithelium in NE-H57 birds was maintained, as it had a normal appearance, whereas NE birds demonstrated a damaged and disintegrated mucosa (Figures 7C, 7D). The villous epithelium is a dynamic bidirectional layer that functions as a frontier barrier entity, accepting or refusing movement of intraluminal particles into adjacent enterocytes and underlying microvasculature. Its integrity is important not only for the digestion and for absorption of nutrients but as the first line of defense against pathogens and toxic molecules. In the present study, histopathologic examination showed that the epithelium of villi was infiltrated by IEIC, which were present also in the lamina propria and crypt epithelium as part of the normal GIT immune system. The IEIC number was significantly elevated in NE-H57 birds (6.52/100 μm villus height) when compared with birds from control and other treatment groups. All infected birds reacted with an increase in immune cell numbers when compared with control birds (Table 5); however, the NE-H57 birds had a better response, which involved other lymphoid follicular structures such as Peyer's patches appearing in the ileal lamina propria to submucosa (Figure 7H).

Histopathologic examination of intestinal tissue from birds exposed to NE confirmed signs of inflammation, such as hyperemia and infiltration of ileal mucosa with lymphocytes and heterophils, swelling or damage of the apical villus, and necrosis of villi (Figures 6D and 7H), whereas in NE-H57 birds, the epithelial structure and function was well maintained. This was confirmed by a lower incidence of pasty vent in NE-H57 birds, compared with that in NE birds. A decrease in pasty vent incidence in chickens indicates a healthier intestinal mucosa, and this is associated with an improvement in nutrient digestibility (Roy et al., 2010). De Cesare et al. (2017) demonstrated that supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) at the recommended dietary dosage of 1 x 10^9 cfu/kg feed significantly reduced the incidence of pasty vent in broiler chickens. In the present study, broiler chicks exposed to NE and fed a diet supplemented with H57 (i.e., NE-H57 birds) had a significant improvement of FCR (FCR was similar to control birds), compared with NE birds. The reduction in production costs through improved FCR is the goal for many broiler producers, and this study demonstrates that H57 can deliver that.

Many commensal bacteria, including probiotics, produce enzymes to breakdown feed substrates to permit bacterial metabolism. These enzymes may also facilitate digestion by the host (Bajagai et al., 2016; Rowland et al., 2018). *B. amyloliquefaciens* is a microorganism that produces large quantities of extracellular enzymes such as amylase, protease, and lipase, as well as some cellulases and xylanases, and can liberate them into intestinal contents (Elshaghabee et al., 2018). This action by the probiotic potentially improves chicken digestion and is one possible mechanism to explain improved performance when birds are fed probiotics (Bajagai et al., 2016). Similar enzymes are routinely added to poultry diets to improve nutrient digestibility and to degrade antinutritive factors (Bedford, 2000; Ravindran, 2013). Lactic acid production is also increased by *B. amyloliquefaciens* metabolism, and this explains the drop in pH observed in this study. A drop in ileum pH has been shown to favor gut colonization by *Lactobacilli* and the suppression of pathogenic bacteria (Wu et al., 2011; Salim et al., 2013).

In conclusion, a damaged intestinal mucosa, decreased nutrient absorption, and impaired growth performance are the main features of subclinical NE. Subclinical NE imposes a significant economic burden on poultry producers and potentially has an impact on human food safety. It exhibits few clinical signs, and it is difficult to diagnose but can spread through flocks, resulting in substantial production losses (Skinner et al., 2010). Data from the present study suggest that dietary addition of H57 improves intestinal health and ameliorates NE signs in birds. Histopathology of the ileum suggests that the probiotic effect appears , in part, because of improved mucosal structure and integrity. The results suggest

that H57 is more effective in birds subjected to an infectious challenge. Birds infected with NE and fed H57 maintained epithelial barrier integrity and had improved feed efficiency.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.05.034.

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