Two different *Clostridium perfringens* strains produce different levels of necrotic enteritis in broiler chickens

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ABSTRACT Subclinical necrotic enteritis (**NE**) is primarily caused by the gram-positive bacterium, *Clostridium perfringens* (***Cp***). The trend towards removal of in-feed antimicrobials and subsequent increased emergence of infection in poultry has resulted in a wide interest in better understanding of the mechanism behind this disease. The virulence of NE, to a large extent, depends on the virulence of *Cp* strains. Thus, this study was to assess how 2 different strains of *Cp* affect performance and gut characteristics of broiler chickens. Ross 308 male broilers ($n = 468$) were assigned to a 2 × 3 factorial arrangement of treatments with antibiotics (Salinomycin at 72 ppm and zinc bacitracin at 50 ppm −, or +) and challenge (non-challenge, *Cp* EHE-NE18, or *Cp* WER-NE36). Oral administration of *Eimeria* oocysts (day 9) followed by inoculation with 1 mL 10^8 CFU *Cp* strains (day 14 and 15) were used to induce NE. Broiler performance was analyzed at day 10, 24, and 35. On day 16, intestinal lesion score and intestinal pH were evaluated and samples of cecal content were analyzed for bacterial counts and short-chain fatty acid concentrations (**SCFA**). Birds in both challenged groups showed higher feed conversion ratio (**FCR**), lower weight gain ($P < 0.001$), increased lesion scores in the jejunum ($P < 0.01$), and reduced pH in the ileum and cecum ($P < 0.01$), compared to the non-challenged birds. They also showed decreased numbers of *Bacillus* spp. ($P < 0.001$), and *Ruminococcus* spp. ($P < 0.01$) in the cecal content. On day 35, the NE36 challenged birds had a lower weight gain ($P < 0.001$) and higher FCR ($P < 0.001$) compared to the NE18 challenged birds. Interestingly, cecal *Lactobacillus* and lactate were increased by the NE challenge ($P < 0.001$), and to a greater extent in birds challenged with NE36 compared to the NE18 strain ($P < 0.001$). This study suggests that *Cp* strains varying in virulence produce different levels of disease in broiler chickens through modulating the gut environment, intestinal microbiota, and SCFA profile to different extents.

Key words: Necrotic enteritis, *Clostridium perfringens*, virulence, microflora, chicken

2019 Poultry Science 98:6422–6432
http://dx.doi.org/10.3382/ps/pez480

INTRODUCTION

Necrotic enteritis (**NE**), an enteric disease caused by the gram-positive anaerobic bacterium, *Clostridium perfringens* (***Cp***), is an economically devastating disease in the broiler industry. The costs of this disease in the global poultry industry is estimated to be approximately US$6 billion per annum in production losses (Wade and Keyburn, 2015; Moore, 2016). This bacterium is present in the intestinal tract of healthy chickens, but toxin producing strains can cause a range of histotoxic infections, enteritis, liver and kidney damage, dermatitis, and gas gangrene (Uzal et al., 2014). Among those, NetB producing strains can infect chickens leading to NE outbreak, either in clinical or subclinical form (Keyburn et al., 2008; Wu et al., 2010). Traditionally, antibiotics have been widely used to control this disease; but the ban in Europe and recent voluntarily phase-out of antibiotics in animal feed in many other countries have made controlling the disease a challenging task.

Extracellular toxins can be produced by different *Cp* strains that are classified as A to G toxinotypes, based on the toxins they produce. All types of the *Cp* strains produce α-toxin (Smedley et al., 2004; Uzal et al., 2010); however, the strains that produce NetB toxin are classified as belong to toxinotype G (Rood et al., 2018). The NetB toxin is recognized as a pore-forming toxin (Savva et al., 2013) and coded by plasmid genomes (Keyburn et al., 2008). It has been reported that the majority of strains isolated from healthy birds do not carry the NetB gene (Lacey et al., 2016), whereas birds infected with NE usually host NetB producing *Cp* (Keyburn et al., 2010). Although NetB is renowned for causing NE, other genomic regions have also been identified as contributors towards the virulence of *Cp* strains (Parreira et al., 2017). The mechanism of virulence of *Cp* on instigating NE is still largely unknown (Lacey et al., 2016; Prescott et al., 2016) but it has been widely recognized that different strains possess different levels of virulence.

Received March 28, 2019.
Accepted July 30, 2019.
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The present study compared the effect of 2 different strains of *Cp* with divergent virulence levels on the severity of resulting NE in broiler chickens. The 2 *Cp* toxinotype G strains selected to challenge birds in the study were EHE-NE18 (NE18) and WER-NE36 (NE36). It has been reported that both NE18 and NE36 strains contain NetB gene and are isolates from NE infected chickens in the field (Lacey et al., 2018). It is also been recognized that NE36 shows higher virulence compared to NE18 (Keyburn et al., 2013). Differences have been identified between NE18 and NE36, such as their ability to bind to specific collagens in the extracellular matrix (Wade et al., 2015). Also, according to their genomic sequences, these 2 strains belong to 2 different pathogenic clades of NE-causing *Cp* strains (Lacey et al., 2018). However, their impact on the chickens with regard to their performance, gut microbial dynamics, and metabolite profile have not been depicted especially under subclinical challenge. We hypothesize that the different *Cp* strains would induce differing levels of NE severity in chickens, through compromised gut health.

MATERIALS AND METHODS

All procedures of this study were reviewed and approved by the Animal Ethics Committee of the University of New England (17/024). All procedures involving the birds, including health, care, and use of laboratory animals, were fulfilled within the Code of Practice for the Use of Animals for Scientific Purposes issued by the Australian Bureau of Animal Health (NHMRC, 2013).

Experimental Procedures, Design, and Diets

Four hundred sixty-eight male broiler chicks (Ross 308) were obtained from Baiada hatchery in Tamworth, NSW, Australia. Birds were weighed and randomly assigned to 36-floor pens with hardwood shavings used as bedding materials. Room temperature was set at 33°C during the first 3 D of the trial and decreased 3°C every week until 24°C was reached at day 21. The lighting, relative humidity, and temperature followed Ross 308 strain guidelines (Aviagen, 2014). The experiment used a completely randomized design with a 2 × 3 factorial arrangement of treatments with antibiotics (−,/+) and challenge (non-challenged/NE18/NE36). The dietary treatments included a control diet (no additive) and this diet supplemented with antibiotics (Salinomycin at 72 ppm and zinc bacitracin at 50 ppm), fed in pellets form for 3 phases, starter (day 1 to 10), grower (day 11 to 24), and finisher (day 25 to 35). The diet was based on wheat and soybean meal and formulated to meet the nutrient requirements recommended by Evonik Industries (Amino Chick 2.0). Diet composition and the analyzed nutrients contents are presented in Table 1. Control diet (no additive) and control diet supplemented with antibiotics were formulated and mixed for the study. All birds had ad libitum access to feed and water throughout the study.

Table 1. Composition and nutrient content of feed.

Ingredient, %	Starter	Grower	Finisher
Wheat	30.0	44.7	34.8
Sorghum	31.0	20.0	30.0
Soybean meal	27.1	19.1	19.0
Canola meal solvent	2.00	5.00	4.5
Meat and bone meal	4.60	5.00	5.00
Canola oil	2.44	3.90	4.80
Limestone	0.67	0.58	0.52
Dical Phos 18P/21Ca	0.65	0.43	0.34
Salt	0.11	0.12	0.13
Na bicarb	0.16	0.13	0.12
UNE VM[1]	0.09	0.09	0.09
UNE TM[2]	0.10	0.10	0.10
Choline Cl 70%	0.04	0.04	0.02
L-lysine HCl 78.4	0.41	0.35	0.32
DL-methionine	0.31	0.24	0.25
L-threonine	0.19	0.15	0.15
Calculated nutrients			
ME kcal/kg	3,025	3,150	3,200
Crude protein %	23.0	22.5	21.4
Isoleucine %	0.98	0.87	0.87
Digestible Arg %	1.31	1.16	1.15
Digestible Lys %	1.27	1.21	1.06
Digestible Met %	0.60	0.61	0.54
Digestible Thr %	0.83	0.73	0.72
Linoleic 18:2%	1.87	2.00	1.95

[1]Vitamin concentrate (DSM Nutritional Products, Wagga Wagga, NSW, Australia) supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2]Trace mineral concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Necrotic Enteritis Challenge

On day 9, challenged groups were inoculated with 1 mL per os field strain of *Eimeria* (Eimeria Pty Ltd, Ringwood, Vic, Australia). Each dose of inoculum consisted of 5,000 sporulated oocysts each of *Eimeria maxima* and *Eimeria acervulina* and 2,500 sporulated oocysts of *Eimeria brunetti* in 1 mL of 1% (w/v) sterile phosphate-buffered saline (**PBS**). Non-challenged groups were inoculated with sterile PBS as a control. Primary poultry isolates of *Cp* strains EHE-NE18 and WER-NE36 containing the toxin NetB (Keyburn et al., 2008) were obtained from CSIRO Livestock Industries, Geelong, Australia. The challenge inocula were freshly prepared by growing the bacterial strains separately in 100 mL of sterile thioglycolate (USP alternative, Oxoid, Australia) with added starch (10 g/L) and pancreatic digest of casein (5 g/L); this was incubated overnight at 39°C. Stock cultures of *Cp* strains were later subcultured in thioglycolate broth followed by cooked meat media (Oxoid, Australia). On day 14 and 15, birds in the challenged group were gavaged with 1 mL per os 10^8 CFU/mL of *Cp* with their respective strains, whereas

the non-challenged birds were gavaged with sterile thioglycolate broth (Rodgers et al., 2015).

Performance Measurements, Sample Collection, pH Evaluation, and Lesion Scoring

Pen and feed weights were measured on day 0, 10, 24, and 35 and mortality was recorded daily. Average body weight (**BW**) gain and feed intake (**FI**) were recorded and FCR was calculated, taking into account bird mortality. Any mortality in the challenge period (day 14 to 16) underwent necropsy to determine if the cause of death is due to NE infection or not.

On day 16, a total of 2 birds from each pen were randomly selected and weighed. All intestinal sections (duodenum, jejunum, and ileum) were excised for lesion scoring, digesta collection, and pH measurements. Intestinal pH values were measured by inserting an EcoScan 5/6 pH meter (Envirosensors spear tip pH probe, Australia) probe into the frontal ileum and cecum sections, respectively. For SCFA analysis, ileal and cecal digesta samples were collected separately into sterile 50 mL containers and stored at −20°C until analysis. Approximately 1 mL of the contents was also placed in sterile 2-mL Eppendorf tubes, snap-frozen in liquid nitrogen and stored at −20°C for DNA extraction. The total length of the small intestine was scored for lesions based on a 0 to 4 scoring system reported by Broussard et al. (1986) where 0 refers to a healthy appearance and 4 a blood-filled intestine with ruptures on the epithelial layer. Two experienced personnel performed lesion scoring with no knowledge of the treatment allocation of the birds.

Cecal Bacterial Enumeration

The DNA was extracted from the frozen cecal samples following the method described by Kheravii et al. (2018). Briefly, approximately 60 mg of frozen cecal samples were added to 300 mg of glass beads (0.1 mm) in a 2 mL Eppendorf tube. A QIAxtractor DNA Reagents, and QIAxtractor DNA plasticware kits (Qiagen, Inc., Doncaster, VIC, Australia) were used for the DNA extraction. Samples were lysed with 300 μL of Qiagen Lysis Buffer with cells disrupted by shaking the tubes in a bead beater mill (Retsch GmbH & Co, Haan, Germany) for 5 min at 30/S frequency. Samples were then placed in a heating block for 2 h at 55°C followed by 5 min centrifuge at 20,000 × *g*. The DNA was extracted using an X-tractor gene automated DNA extraction system (Corbett Life Science, Sydney, Australia). Reagents (DXB, DXW, DXF, and DXE) were placed in their specific locations in the robotics machine together with 200 μL of the lysate transferred automatically into loading block. Then 400 μL of the binding buffer (DXB) was added to the 200 μL lysate and incubated for 6 min, and then 500 μL of the lysed samples were transferred into capture columns and vacuumed at 30 kPa for 3 min. Following this 600 μL DXW was transferred to the capture columns and vacuumed for 30 kPa for 2 min, 600 μL DXF was transferred to the columns and vacuumed at 35 kPa for 1 min, and DNA was dried by vacuuming again at 25 kPa for 5 min. Finally, an elution block was used to elute the extracts by addition of 60 μL DXE and the samples were vacuumed at 30 kPa for 2 min. The resulting DNA samples were measured on a Nanodrop 8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) for assessment of quantity and purity. DNA with ratios of A260/A280 being >1.8 were considered of high purity and were stored at −20°C.

The extracted cecal DNA was diluted 20 times in nuclease-free water and the quantitative real-time polymerase chain reaction (**PCR**) was performed to quantify 6 bacterial groups with a real-time PCR system Rotorgene 6000 (Corbett, Sydney, Australia). The SYBRGreen containing Mix (SensiMix SYBR No-Rox, Bioline, Sydney, Australia) was used for *Bacillus, Bacteroides, Bifidobacteria, Lactobacilli,* and *Ruminococcus* and for *Cp, SensiFAST Probe SYBR No-ROX* (Bioline, Sydney, Australia) was used. The specific 16S rRNA primers used for these groups of bacteria are shown in Table 2.

Cecal SCFAs

The method described by Jensen et al. (1995) was used for the SCFA analysis. In brief, approximately 1 g of cecal digesta was weighed and 1 mL of internal standard (0.01 Methyl-butyric acid) was added. The solution was vortexed and centrifuged for 20 min at 15,000 × *g* at 5°C. An aliquot of 1 mL supernatant was transferred to 8 mL vials, and 0.5 mL of concentrated HCl (36%) and 2.5 mL of ether were added. The mixture was vortexed, centrifuged at 1,000 × *g* for 15 min in 5°C. From the resulting supernatant, 400 μL of it was then mixed with 40 μL N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide. Samples were then vortexed and heated (at 80°C) for 20 min. The vials were then kept in room temperature for 48 h before being analyzed on a Varian CP3400 CX gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). SCFA concentrations are expressed as μmol/g digesta.

Data Analysis

All the data derived were checked for normal distribution prior to conducting statistical analyses. The statistical analysis was carried out with the GLM of SPSS statistics 24 to assess the main effects (challenge and antibiotics) and interactions. Intestinal lesion scoring data was analyzed by the non-parametric Kruskal–Wallis test as the data was not normally distributed. Tukey's mean separation test was used to make pairwise comparisons among treatments when interaction was significant. Statistical significance was declared at

Table 2. Primers used for the qPCR analysis of different bacteria groups.

Target group	Primer/probe sequence	Amplicon length (bp)	Annealing temp. (C°)	Reference
Bacillus spp.	F-GCA ACG AGC GCA ACC CTT GA R-TCA TCC CCA CCT TCC CC GGT	92	63	(Zhang et al., 2015)
Bacteroides spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	108	63	(Layton et al., 2006)
Bifidobacterium spp.	F-GCG TCC GCT GTG GGC R-CTT CTC CGG CAT GGT GTT G	106	63	(Requena et al., 2002)
Clostridium perfringens	F- GCA TAA CGT TGA AAG ATG G R- CCT TGG TAG GCC GTT ACC C TaqMan probe: 5′-FAM-TCA TCA TTC AAC CAA AGG AGC AAT CC-TAMRA-3′	120	60	(Wise and Siragusa, 2007)
Lactobacillus spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	186	63	(Wise and Siragusa, 2007)
Ruminococcus spp.	F-GGC GGC YTR CTG GGC TTT R-CCA GGT GGA TWA CTT ATT GTG TTA A	157	63	(Ramirez-Farias et al., 2008)

Table 3. Performance of chickens in response to the challenge with 2 strains of *Clostridium perfringens* and supplementation of antibiotics during 0 to 35 D of age.

		Performance								
		0 to 10			0 to 24			0 to 35		
Challenge	Antibiotics	BW	FI	FCR	BW	FI	FCR	BW	FI	FCR
Non-challenge	No	284	284	1.010	1,366	1,705	1.247	2,529[a]	3,363	1.330
	Yes	282	282	1.005	1,356	1,697	1.251	2,551[a]	3,436	1.347
NE-18[2]	No	284	285	0.998	1,103	1,431	0.998	2,258[c,d]	3,216	1.424
	Yes	292	291	1.027	1,193	1,527	1.027	2,450[a,b]	3,356	1.373
NE-36[3]	No	293	293	1.023	1,052	1,479	1.023	2,157[d]	3,173	1.475
	Yes	290	291	1.017	1,180	1,528	1.017	2,327[b,c]	3,340	1.435
Main effects										
Antibiotic[1]										
No		288	287	1.009	1,174[b]	1,538	1.323[a]	2,315	3,249[b]	1.403
Yes		287	289	1.012	1,243[a]	1,590	1.292[b]	2,443	3,380[a]	1.385
Challenge										
Non-challenged		283	289	1.007	1,361[a]	1,701[a]	1.250[c]	2,540	3,396[a]	1.338[c]
NE18		288	291	1.002	1,148[b]	1,479[b]	1.308[b]	2,354	3,289[b]	1.398[b]
NE36		290	284	1.025	1,116[b]	1,503[b]	1.362[a]	2,242	3,257[b]	1.455[a]
P- value										
Antibiotic		0.693	0.693	0.666	0.010	0.162	0.032	<0.001	0.002	0.074
Challenge		0.411	0.409	0.091	0.001	0.001	0.001	<0.001	0.016	< 0.001
Antibiotic × challenge		0.610	0.609	0.754	0.084	0.453	0.170	0.013	0.636	0.091

[1]Salinomycin (72 ppm) and zinc bacitracin (50 ppm).
[2, 3]*Clostridium perfringens* strains (10^8 CFU/mL).
[a–d]Means sharing the same superscripts are not significantly different from each other at $P < 0.05$. BW: Body Weight gain (g/bird), FI: Feed intake (g/bird), FCR: Feed conversion ratio.

$P < 0.05$. Correlations between parameters were examined using the Pearson's product moment correlation coefficient test.

RESULTS

Bird Performance

Growth performance of broilers was affected by the strain of *Cp* and the presence of antibiotics at day 24 and 35 of the trial (Table 3). Antibiotics improved BW at day 24 ($P < 0.01$) in all groups. A challenge × antibiotic interactions was observed for BW ($P < 0.05$) at day 35, where antibiotics improved BW in both challenged groups (NE18 and NE36) but did not significantly affect the non-challenged birds. At day 35, antibiotic supplementation as a main effect increased FI regardless of the challenge ($P < 0.01$), while the challenge significantly decreased FI at day 24 ($P < 0.001$) and 35 ($P < 0.05$) in both challenged groups (NE18 and NE36), compared to those in the non-challenged groups. On day 24 and 35, birds challenged with NE36 showed higher FCR ($P < 0.001$) compared to birds challenged with NE18 and both infected groups had higher FCR ($P < 0.001$) compared to the non-challenged birds. However, antibiotics treated birds tended to have a decreased FCR ($P = 0.074$). No significant effect of antibiotic supplementation and challenge was observed

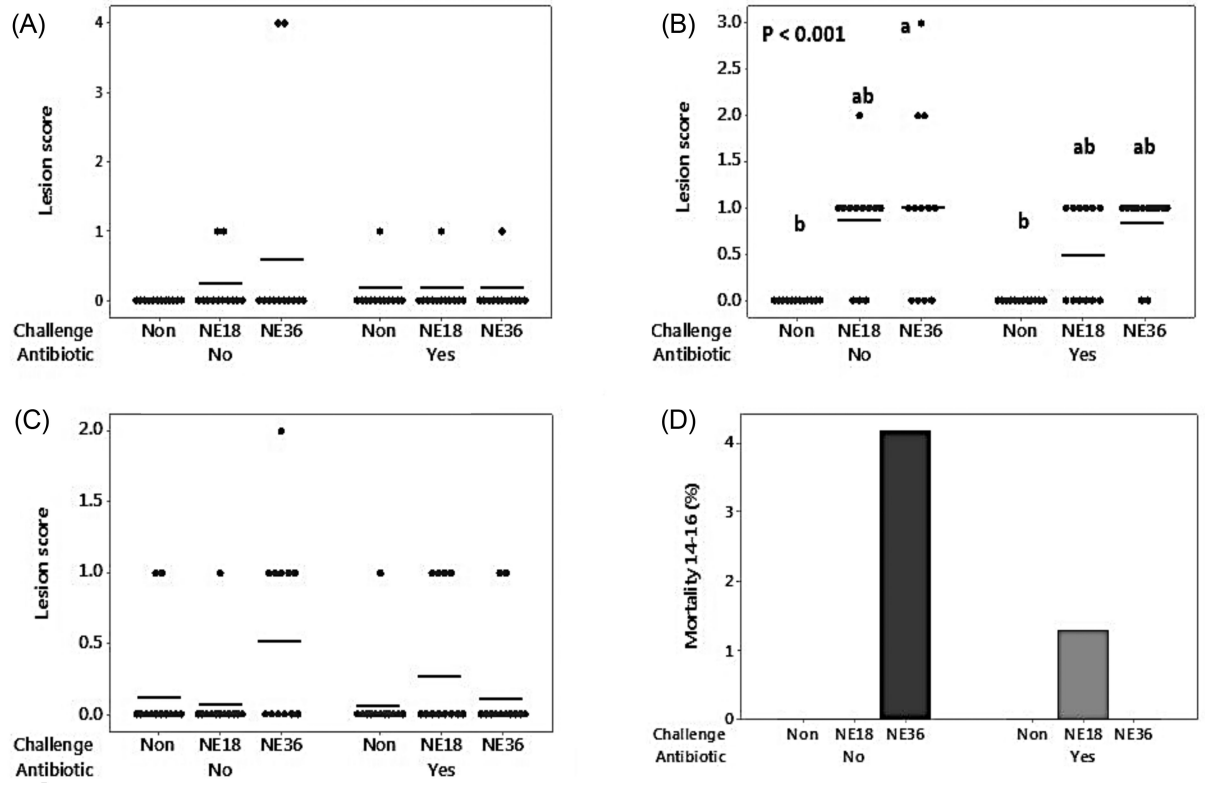

Figure 1. Individual and average intestinal lesion scores of all sampled birds (day 16) challenged with 2 strains of *Clostridium perfringens* (NE18 and NE36). (A) lesion score in duodenum; (B) lesion score in jejunum; (C) lesion score in ileum; (D) Mortality of birds at day 14 to 16.

in the first 10 D of the experiment ($P > 0.05$). No interactions between antibiotic and challenge were observed for FI and FCR in any period of the experiment ($P > 0.05$).

Lesion Score, Intestinal pH, and Mortality

Figure 1 shows gross lesions in 3 sections of the intestines. The NE36 challenged birds fed with control diets produced significantly higher lesions in the jejunum compared to non-challenged groups ($P < 0.01$). The other sections of intestine investigated were not significantly affected by either challenge or antibiotics ($P > 0.05$). During day 14 to 16, a total of 4 mortality were recorded and postmortem examination was performed. One bird from the NE18 challenged group supplemented with antibiotics died at day 15 from NE with damaged intestine. At day 16, a total of 3 birds died from the NE36 challenged groups with no supplemented antibiotics. One bird had an enlarged pale liver that was an untypical NE sign. The other 2 birds had no lesion or other NE-associated signs. The birds had no digesta in the intestine which is the indication that these 2 birds suffered from anorexia. These dead birds were particularly small and weak compared to other birds in the treatment. Statistically, no significant difference was observed in mortality during the challenge period ($P > 0.05$). Cecal and ileal pH was reduced ($P < 0.001$ and 0.01, respectively) in both groups of challenged birds (Table 4).

Table 4. Intestinal pH in responses to the challenge with *Clostridium perfringens* strains NE18 and NE36 and antibiotic supplementation in chickens at day 16.

	pH	
Main effects	Cecum	Ileum
Antibiotic[1]		
No	6.40	5.81
Yes	6.44	5.86
Challenge		
Non-challenge	6.55[a]	5.97[a]
NE18[2]	6.35[b]	5.80[b]
NE36[3]	6.36[b]	5.74[b]
P-value		
Antibiotic	0.338	0.387
Challenge	0.001	0.004
Antibiotic × challenge	0.481	0.926

[1]Salinomycin (72 ppm) and zinc bacitracin (50 ppm).
[2,3]*Clostridium perfringens* strains (10^8 CFU/mL).
[a,b]Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Gut Microflora Changes in Responses to Cp Challenge

Challenged birds had lower *Bacillus* ($P < 0.001$), *Ruminococcus* ($P < 0.01$), and *Bifidobacterium* ($P < 0.05$) counts at day 16 (Figure 2). However, both

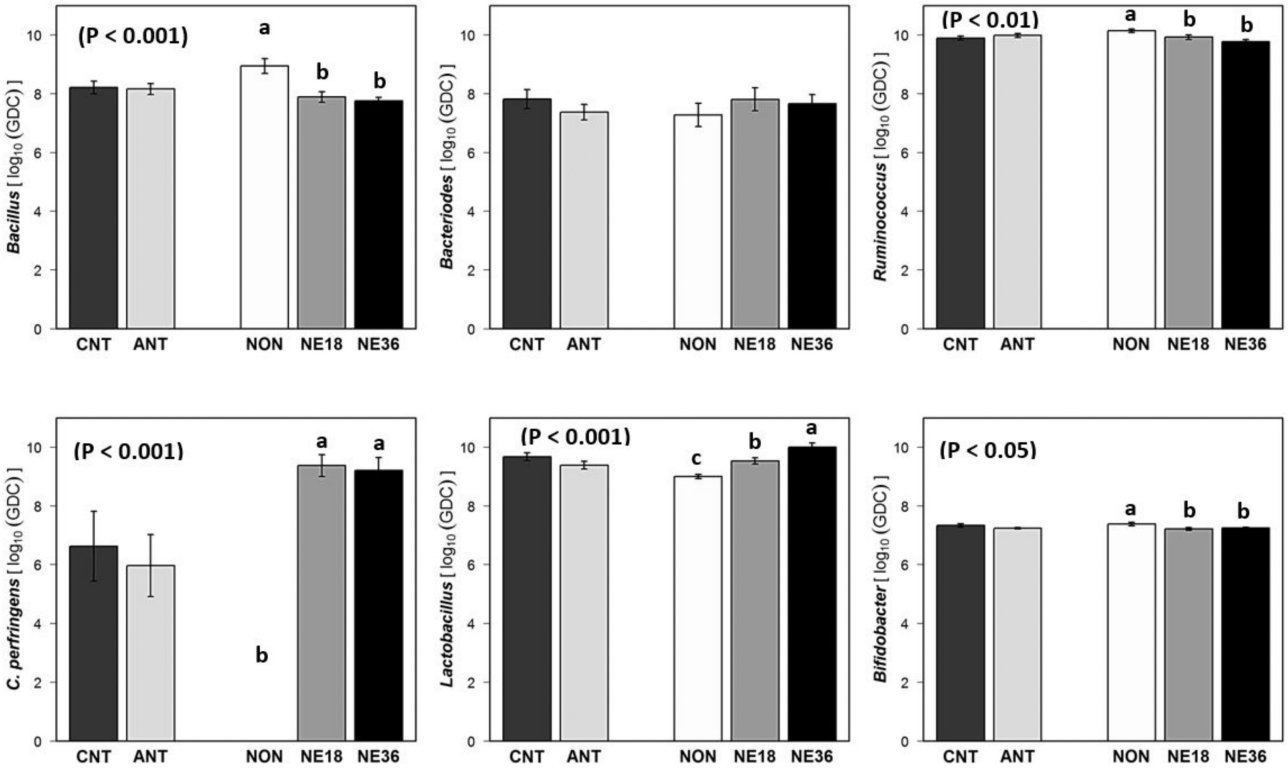

Figure 2. Bacterial levels (Log_{10} copies of respective bacterial genome) in cecal content of birds in response to challenge with NE18 and NE36 strains of *Clostridium perfringens* and antibiotic supplementation at day 16. GDC: Genomic DNA copies/g; CNT: No antibiotic supplementation; ANT: Antibitotic supplementation (Salinomycin at 72 ppm and zinc bacitracin at 50 ppm); NON: Non-challenged; NE18: birds challenged with NE18 strain; NE36: birds challenged with NE36 strain.

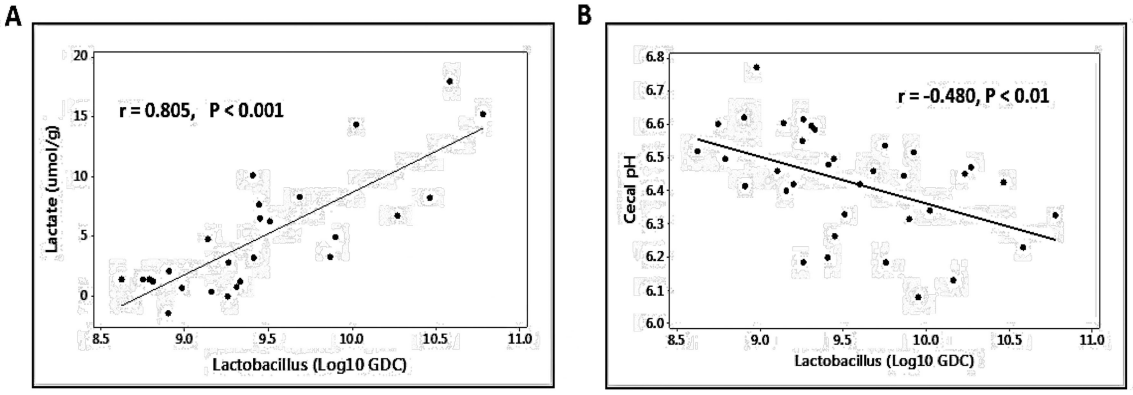

Figure 3. (A) A strong positive correlation between cecal *Lactobacillus* level and lactate concentration in cecum contents; (B) A negative correlation between cecal *Lactobacillus* level and cecal pH.

challenged groups had higher *Cp* ($P < 0.01$) and *Lactobacillus* ($P < 0.001$) counts compared to the non-challenged birds. No significant difference in cecal *Bacteroides* was observed between different treatments. The number of *Lactobacilli* in the ceca tended to be lower in birds fed with the antibiotic supplemented diets compared to those without ($P = 0.057$). No significant antibiotic × challenge was observed for any bacterial species measured ($P > 0.05$). A positive correlation ($r = -0.480$, $P < 0.01$) was observed between cecal Lactobacillus numbers and cecal pH which is illustrated in Figure 3B.

Cecal SCFAs and Correlations with Cecal Bacterial Population

The SCFA concentrations in cecal digesta (μmol/g) are shown in Table 5. Broilers challenged with NE36 strain had increased concentrations of lactate ($P < 0.001$) and propionate ($P < 0.01$) compared to those challenged with the NE18 strain and the control birds. Both NE challenged groups had higher isobutyrate ($P < 0.01$) levels compared to the non-challenged birds. Isovalerate concentrations were significantly higher in NE18 challenged birds compared to the non-challenged

Table 5. Concentration of short-chain fatty acids (μmol/g) in cecal content in responses to the challenge with *Clostridium perfringens* strains NE18 and NE36 and antibiotic supplementation in chickens at day 16.

	Cecal SCFA (μmol/g)							
Main effect	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Lactate	Succinate
Antibiotic[1]								
No	88.7	7.14[a]	0.96	17.2	0.36	1.90	6.29[a]	7.07
Yes	91.3	4.86[b]	0.80	19.5	0.23	1.88	3.26[b]	8.07
Challenge								
Non-challenged	101.0[a]	4.55[b]	0.60[b]	24.6[a]	0.15[b]	1.24[c]	1.35[b]	6.43
NE18[2]	83.4[b]	5.36[b]	1.02[a]	16.1[b]	0.40[a]	1.84[b]	3.38[b]	8.30
NE36[3]	85.4[b]	8.07[a]	1.02[a]	14.6[b]	0.35[a,b]	2.60[a]	10.7[a]	8.92
P-value								
Antibiotic	0.628	0.011	0.149	0.300	0.106	0.922	0.010	0.802
Challenge	0.021	0.005	0.002	0.002	0.027	0.001	0.001	0.368
Antibiotic × challenge	0.916	0.102	0.088	0.921	0.544	0.652	0.333	0.269

[1]Salinomycin (72 ppm) and zinc bacitracin (50 ppm).
[2,3]*Clostridium perfringens* strains (10^8 CFU/mL).
[a–c]Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

Table 6. Pearson correlations between cecal SCFA concentrations or pH and bacterial populations ($\log_{10}$ GDC[1]).

	Acetate	Lactate	Propionate	Butyrate	Valerate	Succinate	Isovalerate	Isobutyrate	Cecal pH
Bacillus	0.058	−0.015	−0.270	0.481**	−0.420*	0.046	−0.479**	−0.495**	0.321
Bacteroides	−0.187	0.008	0.211	−0.214	0.068	−0.078	0.257	0.174	0.097
Ruminococcus	0.196	−0.537*	−0.381*	0.305	−0.447*	−0.173	−0.424**	−0.651	0.300
C. perfringens	−0.486**	0.392**	0.427*	−0.567***	0.573***	0.206	0.501**	0.603**	−0.606***
Lactobacillus	−0.568*	0.806***	0.423*	−0.468**	0.520**	0.291	0.046	0.317	−0.480**
Bifidobacters	0.195	−0.192	−0.192	0.173	−0.174	−0.402**	−0.292	−0.382**	0.239

[1]GDC: genomic DNA copy number.
Stars (*) donate the strength of the significance: $P < 0.001$***, $0.001 < P < 0.01$**, $0.01 < P < 0.05$*.

groups. Furthermore, decreased concentrations of acetate ($P < 0.05$) and butyrate ($P < 0.01$) were observed in the challenged bird. Supplementation of antibiotics reduced propionate ($P < 0.05$) and lactate ($P < 0.05$) concentrations. There was no significant challenge × antibiotic interaction observed for any SCFA measured.

Correlations between bacterial populations and SCFA concentrations or cecal pH were tested and the resuts are shown in Table 6. A strong positive correlation between lactate concentration and *C. perfringnes* ($P < 0.01$) and *Lactobacillus* ($P < 0.001$) population was observed. Acetate and butyrate, on the other hand, showed negative correlation with *C. perfringnes* ($P < 0.01$ and 0.001, respectively) and *Lactobacillus* ($P < 0.05$ and 0.01, respectively). Positive correlations were present for propionate ($P < 0.05$), valerate ($P < 0.001$), isovalerate, and isobutyrate ($P < 0.01$) with *C. perfringens*, and valerate ($P < 0.05$), isovalerate, and isobutyrate ($P < 0.01$) were negatively correlated with *Bacillus*. Furthermore, succinate and isobutyrate were negatively correlated with *Bifidobacteria* ($P < 0.01$ and 0.01, respectively) and lactate, propionate, valerate, and isovalerate negatively correlated with *Ruminococuus* ($P < 0.05$, 0.05, 0.05, and 0.01, respectively). Cecal pH was negatively correlated with only *C. perfringnes* ($P < 0.001$) and *Lactobacillus* ($P < 0.01$).

DISCUSSION

In the current study, the *Cp* strains, NE18 and NE36, introduced different levels of NE severity in broiler chickens. Both strains produced subclinical NE; however, NE36 had a more severe impact on performance resulting in impaired FCR, and decreased BW and FI. The effect of NE36 on lesion scores in the jejunum was significantly higher than the non-challenged birds. Cecal bacterial population and concentration of some cecal SCFAs were altered by at least NE36. The results of the study accept the hypothesis that *Cp* strains with different virulence introduce different levels of NE severity in the chickens by compromising gut health, leading to poorer bird performance.

The introduction of a subclinical NE challenge was successful in this study, based on the observation of typical signs of subclinical NE such as depressed performance, mild lesions in the small intestine, and no NE-related mortality. The disrupted nutrient absorption and utilization inflicted by damaged intestinal mucosa due to *Eimeria* and *Cp* infections could be responsible for reduced BW and impaired FCR as has been shown before (Attia et al., 2012; Rodrigues et al., 2017; Xue et al., 2018). The reduced FI in NE challenged birds is believed to be correlated to the immune system activation, as activated cytokines can reduce FI and consequently lower BW in infected birds

(Dantzer, 2004). In this study, the NE36 strain significantly reduced BW and increased FCR compared to the NE18 strain. The antibiotic × challenge interaction observed on BW confirmed that antibiotic supplementation could only positively affect BW in the presence of NE challenge. The general lack of an antibiotic effect is in agreement with other studies that have shown that well-nourished healthy broilers reared under clean and disinfected conditions have minimal response to antibiotic supplements (Toghyani et al., 2010; Erener et al., 2011). Positive effects of antibiotics could also be due to the suppressive effects of salinomycin on *Eimeria* decreasing the intestinal damage caused by oocytes, thus reducing the chance of birds to be predisposed to NE. Nevertheless, the numeric improvement in BW and FCR and a significant increase in FI in chickens fed with antibiotics confirms the overall positive impact of antibiotic supplementation, as illustrated by a number of other studies (Ocak et al., 2008; Crisol-Martínez et al., 2017).

It is widely accepted that intestinal microflora and their metabolic activity have a significant impact on broiler health and performance. Gut microbiota are key regulators of immune functions and inflammatory responses during disease outbreaks (Prenderville et al., 2015). Thus, they play a critical role in the occurrence and severity of the disease and resilience of birds towards the infections. In the present study, it is interesting that higher levels of cecal *Lactobacillus* were observed in the challenged birds compared to the control. *Lactobacillus* is the most abundant bacterial group accounting for 98% of total bacteria in the upper gastrointestinal tract (GIT) (Gong et al., 2007; Stanley et al., 2012), and some strains of the species are widely used as probiotics to inhibit the growth of *Cp* through the production of organic acids and bacteriocins (Caly et al., 2015). Intuitively, one would expect that increased *Lactobacillus* in the intestine may indicate a healthier gut, whereas it does not seem to be the case according to the findings of the current study together with others. It is therefore proposed that the increase of *Lactobacillus* in challenged birds could be attributed to the following factors: (1) possible perturbations of intestinal nutrient supply by the disease challenge and complex nutritional requirements by bacteria including *Lactobacillus*; (2) possible boost of *Lactobacillus* in the intestine by the recovery of gut health of the birds following NE challenge; and (3) over-influx of upper intestinal content, i.e., in ileum, where *Lactobacilli* are the main bacterial population, to the ceca caused by NE challenge. The microbiota composition produced by challenge could affect the available nutrients for bacteria including *Lactobacillus* thus the dynamics of its population in the intestine (Stanley et al., 2012). On the other hand, Latorre et al. (2018) proposed that an increased abundance of *Lactobacillus* may be related to the rapid recovery of morbid birds in the challenged group. This may be the case in some circumstances as the recovery of the birds from NE infection is very fast with lesions caused by NE healing within 5 D of *Cp* challenge (Keerqin et al., 2017).

Intestinal microbiota modulation also changes the pattern of SCFA concentrations in the gut, which can affect intestinal function and integrity (Meimandipour et al., 2010). As mentioned earlier, an over-influx of upper GIT content to the ceca in NE challenged birds may partly answer the question as to why there was an abundance of *Lactobacillus* in NE challenged birds. *Lactobacillus* is one of the lactic acid producing bacteria and the main end product of these bacteria is lactic acid (Garvie, 1980; Alvarez-Sieiro et al., 2016) that has the ability to produce peristalsis, which in turn can increase nutrient transit rate through the intestine (Saunders and Sillery, 1982). Therefore, the upper GIT content may bring more bacteria, mainly species of *Lactobacillus,* into the ceca. In the present study, the NE36 challenged birds had the highest concentration of lactate, which is in accordance with the changes of *Lactobacillus* abundance. Additionally, among the correlations observed between bacteria population and the SCFAs concentrations *Lactobacillus* and Lactate showed the strongest positive correlation (Figure 3A) that could indicate the relationship between these two parameters. Other studies have previously observed increased abundance of *Lactobacillus* in NE challenged birds (M'Sadeq et al., 2015; Lin et al., 2017; Latorre et al., 2018). Conversely, it has also been reported that NE challenge leads to lower abundance of *Lactobacillus* (Qing et al., 2017; Kheravii et al., 2018) or the overall *Lactobacillus* numbers remain stable (Mikkelsen et al., 2009; Wu et al., 2010; Stanley et al., 2012). Our findings, together with those from other studies (Engberg et al., 2000; Crisol-Martínez et al., 2017), support the notion of a suppressed growth rate associated with the upsurge of *Lactobacillus* numbers. The current study seems to suggest that the level of *Lactobacillus* in GIT especially in the case of NE challenge is not necessarily the indication of the health status of chickens, as one would expect. However, with the converse results presented in different studies, further research is needed to understand the relationship between NE and *Lactobacillus* population.

Ruminococcus is also one of the most dominant bacteria prevalent in the ceca (Park et al., 2017). It plays an important role in digestion and can produce bacteriocins that may control the growth of *Cp* (Crost et al., 2011). In this study, the cecal *Ruminococcus* population decreased in the NE challenge birds. We speculate that lower numbers of this bacterium could be a sign of impaired digestion and immune defense in the gut. *Ruminococcus* produces butyrate as a primary end product (Takahashi et al., 2016). The role of this fatty acid in energy metabolism and intestinal epithelial cell proliferation is well documented (Rinttilä and Apajalahti, 2013); it can increase arterial blood flow that is linked to improved nutrient absorption (Whitehead et al., 1986), stimulates host defense peptides, and has anti-inflammatory actions on the intestine

epithelium (Sunkara et al., 2011; Celasco et al., 2014). This suggests that lower levels of *Ruminococcus* and thus limited available butyrate in the intestine may have led to poorer performance in the challenged birds.

The extracellular toxins produced by *Cp* can disrupt the phospholipid bilayer in cells, causing inundation of ions, which, in turn, may cause osmotic balance change and consequential cell lysis (Yan et al., 2013). This intestinal damage will affect the ability of birds to uptake nutrients, instead of making them available for the use of microorganisms in the intestine (Choct, 2009). It is suggested that different strains of *Cp* affect the performance differently through modulating gut microbiota and immunity of chickens under challenge. The more virulent strains show a more severe impact on performance and negatively affected microbiota compared to the less virulent strains. However, it should be noted that these negative results can also be partially due to the coccoidal infections prior to the *Cp* challenge, as coccidia is capable of producing damage in the gut that can affect BW and FCR by microbiota modulation, nutrient uptake, and immune responses (Tan et al., 2014). Essentially, the administration of *Eimeria* spp. in the NE challenge model was to predispose birds for the effective infection by *Cp*. Therefore, it is expected that coccoidal infection would affect the birds negatively. However, more detailed investigations on the mechanism that underlies the virulence and how the *Cp* strains differ in their impact on the gut health and integrity thus the performance of the chickens is warranted.

In conclusion, the broilers infected with NE18 and NE36 showed subclinical NE, and NE36 impaired performance more severely than NE18. It has been speculated that the impact of the *Cp* strains on the disease and performance of the broiler chickens is due to their influence on the gut microfloral dynamics and thus the profile of bacterial metabolites such as SCFAs in the intestine. The changes of particular bacterial groups by the *Cp* challenge may be the indications of gut health under certain conditions. However, further data need to be collected for robust proof of how these bacterial changes affect the intestinal health of chickens.

ACKNOWLEDGMENTS

The authors thank Petrina Young of Eimeria Pty Ltd for providing *Eimeria* species, Robert Moore for providing *Clostridium perfringens* EHE-18 and WER-36 strains, and Shuyu Song for her help and guidance with the lab work.

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