Differential expression of intestinal genes in necrotic enteritis challenged broiler chickens with 2 different *Clostridium perfringens* strains

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ABSTRACT The primary cause of necrotic enteritis (**NE**) disease in chickens is the NetB-positive *Clostridium perfringens* bacterium. Many factors are known to affect the severity of NE in the challenge models of broiler chickens, and one of these factors is the virulence of *C. perfringens* strain. This study was conducted to evaluate the effect of 2 pathogenic *C. perfringens* strains in a NE challenge model on gut health and mRNA expression of genes encoding apoptosis, tight junction, immunity, and nutrient transporters in broilers. Day-old Ross-308 male broilers ($n = 468$) were allocated in a 2×3 factorial arrangement of treatments with in-feed antibiotics (no or yes) and challenge (Non, *C. perfringens* strain NE18, and *C. perfringens* strain NE36) as the factors. The birds in the challenged groups were inoculated with *Eimeria* species on day 9 and with a fresh suspension of *C. perfringens* NE18 or NE36 on day 14 and 15. Sample collection was performed on 2 birds of each pen on day 16. Necrotic enteritis challenge, impaired feed conversion ratio during day 0 to 16 compared with the control group where the effect of the NE36 challenge was more severe than that with NE18 ($P < 0.001$). The mRNA expression of mucin-2, immunoglobulin-G, occludin ($P < 0.001$), and tight junction protein-1 ($P < 0.05$) genes were downregulated in both challenged groups compared with the nonchallenged counterparts. Antibiotic supplementation, on the other hand, increased weight gain, and feed intake in all challenged birds ($P < 0.01$), but upregulated mucin-5ac and alanine, serine, cysteine, and threonine transporter-1 ($P < 0.05$) only in the NE18 challenged birds. The challenge with NE36 significantly upregulated caspase-8 and claudin-1 ($P < 0.001$), but downregulated glucose transporter-2 ($P < 0.001$) compared with the NE18 challenge. These results suggest that NE challenge is detrimental to the performance of broilers through compromised intestinal health, and different *C. perfringens* strains can affect the severity of the disease through modulating the expression of intestinal genes encoding proteins responsible for apoptosis, gut integrity, immunity, mucus production, and nutrient transporters.

Key words: necrotic enteritis, *Clostridium perfringens*, virulence, strains, gene expression, broiler

2021 Poultry Science 100:100886
https://doi.org/10.1016/j.psj.2020.11.063

INTRODUCTION

Clostridium perfringens is known to be the causative agent of necrotic enteritis (**NE**) in chickens. This gram-positive anaerobic bacterium is a resident in the intestinal tract of both humans and livestock and usually does not cause disease (Cooper and Songer, 2010). However, with the removal of in-feed antibiotics from broiler diets, there has been an immense increase in NE incidence in poultry farms in Europe and the Unites States (McDevitt et al., 2006). The cost of NE worldwide has been estimated to be 6 billion dollars annually, which includes not only direct loss due to broiler mortality but also reduced performance and management costs (Wade and Keyburn, 2015). The proliferation of *C. perfringens* along with one or more predisposing factors in the gut mediates the disease by the production of, mostly, extracellular protein toxins (Craven, 2000).

Recently, numerous developments have been made for understanding the pathogenesis of necrotic enteritis in broilers (Kaldhusdal et al., 2016). In the presence of predisposing factors, the strains that produce the pore-forming NetB toxin can lead to the production of necrotic enteritis, as this toxin is essential for the disease to occur when predisposing factors are present (Rood

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Received May 12, 2020.

Accepted November 23, 2020.

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et al., 2018). Recent reports have shown that the pathogenesis and virulence of *C. perfringens* are also controlled by other genes or loci (Coursodon et al., 2012; Zhou et al., 2017) and these factors could contribute to the different severity levels of the NE disease.

The importance of the intestinal tract and its critical role in nutrient absorption and immune responses is evident (Lan et al., 2005). The mucosal barrier mechanism in the small intestine serves as the first line of defense and can maintain an essential barrier to microbial invasion and protect the intestinal epithelial cells (Elphick and Mahida, 2005). Mucin proteins, such as mucin-2 (**MUC2**) and mucin-5ac (**MUC5ac**) maintain a suitable mucous layer as this layer is continually sloughed off by intestinal movements of microbial-derived factors (Horn et al., 2009). Immunoglobulin proteins such as immunoglobulin-M (***IgM***) and immunoglobulin-G (**IgG**) are present in the enterocyte brush border and are delivered to the mucus layer to participate in immune responses and clearance of antigens (Hansen et al., 2005). Furthermore, intestinal absorbing epithelial cells are strongly connected by tight junction (**TJ**) proteins such as claudin-1 (**CLDN1**), occludin (**OCLN**), and tight junction protein-1 (**TJP1**). The function of these proteins is necessary for controlling permeability of the paracellular pathways (Furuse et al., 2002; Elkouby-Naor and Ben-Yosef, 2010). On the other hand, although apoptosis (cell death) usually occurs during development and aging, it can also happen in defense mechanisms such as cell damage caused by disease or toxic agents (Norbury and Hickson, 2001). It is known that activation of caspase family correlates with the onset of apoptosis and cell death (Cohen, 1997). Brush border enzymes, Na^+-dependent neutral amino acid transporters, Na^+-dependent neutral/cationic amino acid exchanger and, glucose transporter-2 (**GLUT2**) in the intestinal epithelium are closely associated with intestinal nutrient absorption capacity (Uldry et al., 2002; Hediger et al., 2004; Fotiadis et al., 2013).

Brush border enzymes include aminopeptidase N (**APN**) and sucrase-isomaltase (**SI**); Na^+-dependent neutral amino acid transporters include B^0AT and alanine, serine, cysteine, and threonine transporter-1 (**ASCT1**), and Na^+-dependent neutral/cationic amino acid exchangers include Y + L amino acid transporter-1 (**y^+LAT1**) and Y + L amino acid transporter-2 (**y^+LAT2**).

The effect of the 2 *C. perfringens* strains, ERE-NE18 (**NE18**) and WER-NE36 (**NE36**), on broiler performance, gut microbiota, and intestinal short-chain fatty acid concentrations have been previously reported. Gharib-Naseri et al. (2019) observed a greater negative impact of NE36 challenge on the performance and microbiota profile of broilers compared with NE18 strain. Wade et al. (2015) reported that these 2 strains may have differences in their ability to bind to specific collagens in the extracellular matrix. The expression level of intestinal genes was examined in the present study to determine the changes generated in the intestinal tissue of broilers infected with NE at molecular level. We hypothesized that NE challenge is detrimental for the chickens on their performance through compromised gut health such as intestinal gut integrity, morphology, and regulation of genes coding–related proteins, which may underlie the mechanism of infection by causative agent *C. perfringens* together with the predisposing factors. In addition, the strains of *C. perfringens* used in the challenge may play important role in the severity of the infection.

MATERIALS AND METHODS

The following experimental protocol was approved by the Animal Ethics Committee (authority no.: AEC17-024) of the University of New England, Armidale, NSW, 2351, Australia. The protocol was carried out in accordance with the guidelines specified in the Australian Code for the Care and Use of Animals for Scientific Purposes eighth edition 2013. Briefly, Ross-308 were reared on floor pens with feed and water were provided *ad libitum* for chickens (Gharib-Naseri et al., 2019). Lighting, relative humidity, and the temperature were set in accordance with Ross-308 strain guidelines (Aviagen, 2014).

Experimental Design and Diets

A total of 468 day-old male Ross-308 chickens were obtained from Baiada hatchery in Tamworth, NSW, Australia. On arrival, all birds were weighed and assigned to 36-floor pens with 13 birds in each pen. This experiment compromised 6 treatment groups and was designed as a 2 × 3 factorial arrangement of treatments with antibiotics (no or yes) and challenge (Non, *C. perfringens* strain NE18, or *C. perfringens* strain NE36). The control diet (no additive) and a diet supplemented with the antibiotics salinomycin sodium (72 ppm active, Sacox Huvepharma, Sydney, Australia) and zinc bacitracin (50 ppm active, Albac 150, Pfizer Australia Pty Ltd., Sydney, NSW, Australia). Antibiotics were added in diets from the first day of the experiment. All diets were cold-pelleted (65°C–70°C) and chickens were fed starter diets from day 0 to 10, and grower diets from day 11 until the birds were sampled at day 16.

Necrotic Enteritis Challenge

All chickens in both NE challenge groups underwent a series of inoculations to induce NE challenge. The University of New England NE challenge procedure (Rodgers et al., 2015) was followed to introduce subclinical NE disease. In brief, all challenged birds were orally gavaged with 1 mL/bird field *Eimeria* strains (*Eimeria acervulina* 5,000 oocytes/mL, *Eimeria maxima* 5,000 oocytes/mL, *Eimeria brunetti* 2,500 oocytes/mL) on day 9; chickens in the nonchallenged groups were inoculated with 1 mL of sterile PBS. Primary poultry isolates of the 2 *C. perfringens* strains (EHE-NE18 and WER-NE36) 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 *C. perfringens* strains were later subcultured in thioglycolate broth followed by cooked meat media (Oxoid, Australia). Fresh inoculums of each strain containing approximately 10^8 CFU/mL *C. perfringens* were separately prepared and 1 mL of the inoculums were inoculated to the chickens in accordance with their challenge groups on day 14 and 15. Chickens in the nonchallenged groups were gavaged with sterile thioglycolate medium as a sham treatment.

Sample Collection

On day 16, 2 birds from each pen were randomly selected and euthanized to collect blood for the measurement of fluorescein isothiocyanate-dextran (**FITC-d**), and jejunal tissue for gene expression assay and histomorphological parameters. Blood collection was performed by electrical stunning the birds and immediately collecting blood from the jugular vein before decapitation. After dissecting chickens, 5 cm of the proximal jejunum tissue was excised. Approximately 2 cm of the tissue was separated, flushed with PBS (4°C) and collected in 2 mL Eppendorf tubes filled with RNA later (Qiagen, Germany) and kept at 4°C for 24 h, and then stored in −20°C until required. The rest section of the sample was flushed with PBS and kept in 10% buffered formalin until required for histology processing.

Detection of Fluorescein Isothiocyanate-Dextran in Serum

Two chosen birds from each pen were inoculated with 1 mL FITC-d (4.17 mg/kg bird, average molecular weight 4,000; Sigma–Aldrich Co., Australia), 2.5 h before euthanization and blood collection as described above (Vicuna et al., 2015). Blood samples were kept in room temperature for approximately 3 h followed by 15-min centrifugation at 1,000 × g to separate red blood cells from serum. The supernatant serum samples were collected and fluorescent levels in serum samples were measured with an excitation wavelength of 485 nm and an emission wavelength of 528 nm on a Synergy HT, Multimode microplate reader (SpectraMax M2e, Molecular Devices, CA) as explained by Kuttappan et al. (2015). Levels of fluorescence in the samples were converted to respective FITC-d microgram per milliliter of serum based on a calculated standard curve obtained from known levels of FITC-d.

Histology

Jejunum sections of sampled birds were fixed in formalin solution for at least 3 d. The fixed tissue samples were processed in an automated tissue processor (Thomas Optical and Scientific Co., Melbourne and Sydney, Australia) and embedded in paraffin (Leica EG 1160; Leica Microsystems, Bensheim, D-64625, Germany). The tissue sections were cut at 5 μm using a microtome (Leitz 1516; Leica Microsystems, Bensheim, D-64625, Germany), mounted on slides with DPX Mountant for histology (Aldrich Chemical Company, Inc., Milwaukee, WI 53255, MO). Staining and analysis of the samples were according to M'Sadeq et al. (2015).

RNA Extraction and cDNA Synthesis

Total RNA from each jejunal sample was extracted using TRIsureTM (Bioline, Sydney, Australia) following the manufacturer's instructions. All RNA samples were purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity and purity of the samples were measured with NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and integrity with the Agilent 2100 Bioanalyzer, 6000 Nano kit. (Agilent Technologies, Inc., Waldron, Germany). The samples were accepted as high quality if the value of 260/230 was >2.0, 260/280 value was between 2.0 and 2.2, and the RIN number of each sample was higher than 7. The extracted RNA of each sample was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The Rotor-Gene 6000 real-time PCR machine (Corbett, Sydney, Australia) was used to convert the RNA into cDNA. The cDNA was diluted 10 times with nuclease-free water and stored at −20°C until required.

Real-Time Quantitative PCR

The primers used in this study were either sourced from literature or newly designed as shown in Table 1. All primers were analyzed specificity by PCR with a subpopulation of samples and fragments separated on Agilent 2100 Bioanalyzer using Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany). Quantitative PCR was performed in duplicates using an SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, Australia) with Rotor-Gene 6000 real-time PCR machine (Corbett Research, Sydney, Australia). The PCR reaction was performed in a volume of 10 μL containing 5 μL of 2 × SensiFAST, 400 mM of each primer and 2 μL of 10 × diluted cDNA template. The relative quantity of mRNA of the target genes were calculated by qBase + version 3.0 (Biogazelle, Zwijnbeke, Belgium) software with *YWHAZ* and *HMBS* as reference genes that were optimized from 10 widely used housekeeping genes before the analysis of target genes. The qBase + applied an arithmetic mean method to transform logarithmic Cq value to linear relative quantity using the exponential function for relative quantification of target genes (Vandesompele et al., 2002; Hellemans et al., 2007). The output data were exported to SPSS statistical version 22 (IBM SPSS, UK) statistical software for further analysis.

The genes used for expression analysis in the jejunal tissue are as listed: caspase-1, 2, 3, 6, 8, and 9 (*CASP1*,

Table 1. Sequences of primers used for quantitative real-time PCR.

Gene	Accession N°	Sequence	Size (pb)	Annealing T°	Reference
CASP1	AF031351.1	F-ACATATACCAGCCACGGGAGA R-CATTGTAGCCCAGCCCTTCT	141	60	This study
CASP2	NM_001167701.1	F-CAGCGATACCACCAGGAAGC R- GCTTCCAGACTTCGCCTGTATC	144	60	This study
CASP3	NM_204725.1	F-TGGTGGAGGTGGAGGAGC R- GTTTCTCTGTATCTTGAAGCACCA	110	62	This study
CASP6	NM_204726.1	F- AAGCCTCTCGGGATGACTACA R- TCACCTCGACATGCCTGAAT	193	60	This study
CASP8	NM_204592.2	F-GGAGCTGCTATCGGATCAAT R-GGAGCTGCTCTATCGGATCAAT	126	60	This study
CASP9	XM_424580.5	F- GGAATGAGGACGAGCCAGAC R- TGTCTGACACCCGAAGTAGCAT	198	60	This study
BCL2	NM 205339	F-CACCTGGATGACCGAGTACC R-GTCCAAGATAAGCGCCAAGA	191	60	(Zhao et al., 2013)
IgG	X07174.1	F: ATCACGTCAAGGGATGCCCG R: ACCAGGCACCTCAGTTTGG	118	60	(Zhao et al., 2013)
IgM	X01613.1	F: GCATCAGCGTCACCGAAAGC R: TCCGCACTCCATCCTCTTGC	98	60	(Zhao et al., 2013)
MUC2	XM 001234581.3	F- CCCTGGAAGTAGAGGTGACTG R- TGACAAGCCATTGAAGGACA	143	60	(Fan et al., 2015)
MUC5ac	XM 003641322.2	F- AAGACGGCATTTATTTCTCCAC R- TCATTACCAACAAGCCAGTGA	244	60	(Fan et al., 2015)
OCLN	NM_205128.1	F- ACGGCAGCACCTACCTCAA R- GGGCGAAGAAGCAGATGAG	123	60	(Du et al., 2016)
TJP1	XM_413773.4	F-GGATGTTTATTTGGGCGGC R-GTCACCGTGTGTTGTTCCCAT	187	60	(Zanu et al., 2020)
CLDN1	NM_001013611.2	F-CTTCATCATTGCAGGTCTGTCAG R-AAATCTGGTGTTAACGGGTGTG	103	60	(Zanu et al., 2020)
APN	NM_001013611.2	F-AATACGCGCTCGAGAAAACC R-AGCGGGTACGCCGTGTT	70	60	(Gilbert et al., 2007)
ASCT1	XM 001232899.4	F-TTGGCCGGGAAGGAGAAG R-AGACCATAGTTGCCTCATTGAATG	63	60	(Paris and Wong, 2013)
$b^{0,+}AT$	NM_001199133.1	F-CAGTAGTGAATTCTCTGAGTGTGAAGCT R-GCAATGATTGCCACAACTACCA	88	60	(Gilbert et al., 2007)
$B^{0}AT$	XM_419056.5	F-GTGTTTGGAACCCTAAATACGAGG R-TAGCATAGACCCAGCCAGGA	72	60	(Kheravii et al., 2018)
GLUT2	NM_207178.1	F-TGATCGTGGCACTGATGGTT R-CCACCAGGAAGACGGAGATA	171	60	(Kheravii et al., 2018)
LAT1	KT876067.1	F-GATTGCAACGGGTGATGTGA R- CCCCACACCCACTTTTGTTT	70	60	(Gilbert et al., 2007)
ATP1A1	NM_205521.1	F-GTCAACCCGAGGGATGCTAA R-ACTGCTACAATGGCACCCTG	179	60	(Kheravii et al., 2018)
PepT2	NM_001319028.1	F-TGACTGGGCATCGGAACAA R-ACCCGTGTCACCATTTTAACCT	63	60	(Paris and Wong, 2013)
SI	XM_015291762.1	F-GCTTTAAG↓ATGGGCAAGAGGAAG R- CCACCACCAGGCAAAAGAGG	65	60	(Kheravii et al., 2018)
$y^{+}LAT1$	XM_418326.5	F-TACTGAGGCTGACTGGAGGAA R- ACGACGTACAGCACAAT↓ATCTGG	227	62	(Kheravii et al., 2018)
$y^{+}LAT2$	NM_001005832.1	F-GCCCTGTCAGTAAATCAGACAAGA R-TTCAGTTGCATTGTGTTTTGGTT	82	60	(Gilbert et al., 2007)
HMBS	XM 417846.2	F: GGCTGGGAGAATCGCATAGG R: TCCTGCAGGGCAGATACCAT	131	60	(Yin et al., 2011)
YWHAZ	NM_001031343.1	F- TTGCTGCTGGAGATGACAAG R- CTTCTTGATACGCCTGTTG	61	60	(Bagés et al., 2015)

CASP2, *CASP3*, *CASP8*, *CASP9*), B-cell lymphoma-2 (***BCL2***), *IgG* and *IgM*, *MUC2* and *MUC5ac*, occludin (*OCLN*), *TJP1*, *CLDN1*, *APN*, *ASCT1*, $b^{0,+}$amino acid transporter ($b^{0,+}AT$), neutral amino acid transporter ($B^{0}AT$), GLUT2, large neutral amino acid transporter-1 (*LAT1*), ATPase Na+/K+ transporting subunit alpha-1 (*ATP1A1*), peptide transporter-2 (***PepT2***), *SI*, $y^{+}LAT1$, and $y^{+}LAT2$.

Statistical Analysis

All the data derived were evaluated for normal distribution before statistical analyses. Data were analyzed in accordance with a 2 × 3 factorial arrangement of treatments, using the general linear model procedure of SPSS 24 package to assess the main effects of challenge and antibiotics, and their interactions. Tukeys' test was used to perform pairwise comparisons between means. Significant values were based on $P < 0.05$; P values between 0.051 and 0.10 were reported as a tendency of significance.

RESULTS

Growth Performance

Table 2 shows the effect of 2 *C. perfringens* strains (NE18 and NE36) on the performance of birds from

Table 2. Performance results of broiler chickens under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36), from day 0 to 16.

Challenge	Antibiotic[1]	BW	FI	FCR
Non	No	624[a]	730[a]	1.170
	Yes	620[a]	717[a]	1.157
NE18	No	453[d]	595[b]	1.313
	Yes	525[b]	660[b]	1.257
NE36	No	440[d]	598[c]	1.359
	Yes	488[c]	634[c]	1.301
Main effects				
Antibiotic	No	506	641	1.281[a]
	Yes	545	671	1.238[b]
Challenge	Non	622	724	1.163[c]
	NE18	490	628	1.285[b]
	NE36	646	616	1.330[a]
P-value				
Antibiotic		<0.001	0.004	0.001
Challenge		<0.001	<0.001	<0.001
Antibiotic × challenge		0.002	0.008	0.225

[a,b,c]Means with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

Abbreviations: BW, body weight gain (g/bird); FI, feed intake (g/bird); FCR, feed conversion ratio; Non, nonchallenged; NE18, *C. perfringens* strains ERE-NE18, 10^8 CFU/mL; NE36, *C. perfringens* strain WER-NE36, 10^8 CFU/mL.

[1]Antibiotic: salinomycin (72 ppm) and zinc bacitracin (50 ppm).

day 0 to16. An antibiotic × challenge interaction was observed for weight gain ($P < 0.01$) and feed intake ($P < 0.01$), where antibiotics only increased weight gain and feed intake in challenged birds. As expected, the FCR was impaired in both challenge groups with NE18 and NE36 ($P < 0.001$). In addition, birds challenged with the NE36 strain had higher FCR than those in the NE18 challenge group. No significant antibiotics × challenge interaction was observed for FCR.

Gut Integrity and Morphology

As shown in Table 3, gut integrity analysis showed higher FITC-d concentrations ($P < 0.001$) in the serum of the birds challenged with NE compared with nonchallenged birds. Furthermore, a significant reduction in gut permeability ($P < 0.05$) was also observed in birds fed with antibiotics. Results of the villus height, crypt depth, and villus/crypt ratio of the jejunum at day 16 are presented in Table 3. Challenged birds showed lower villus height ($P < 0.001$), deeper crypt ($P < 0.001$), and lower villus height/crypt depth ratio ($P < 0.001$) than nonchallenged birds. No antibiotic × challenge interaction was observed for all the variables described. Figure 1 illustrates the histology of jejunum tissue in chickens.

Upregulation of CASP3 and CASP8 by NE Challenge

The mRNA expression of 6 genes related to apoptosis in the jejunum was examined to investigate their responses to the treatments (Figure 2). The genes *CASP3* (Figure 2C) and *CASP8* (Figure 2D) were upregulated in both challenged groups ($P < 0.001$) relative to nonchallenged birds, and an antibiotic × challenge interaction was observed in the expression of *CASP3* ($P < 0.05$), where expression of *CASP3* was reduced by antibiotics only in nonchallenged birds (Figure 2E). Antibiotics also downregulated the expression of *CASP8* ($P < 0.05$) regardless of the challenge. The NE challenge upregulated *CASP8* ($P < 0.001$) in both NE18 and NE36 groups, whereas birds in the NE36 group had a significantly higher expression of *CASP3* and *CASP8* ($P < 0.001$) than birds challenged with NE18. The mRNA expression of *CASP1*, *CASP2*, *CASP9*, and *BCL2* were not affected by the *C. perfringens* strains or antibiotic supplementation.

Downregulation of OCLN and TJP1 and Upregulation of CLDN1 by NE Challenge

As shown in Figure 3, both challenge groups showed downregulated expression of *TJP1* ($P < 0.05$) and *OCLN* ($P < 0.001$) in chickens (Figures 3B and 3C, respectively). *CLDN1*, on the other hand, was upregulated ($P < 0.001$) by both NE18 and NE36 strains and birds challenged with NE36 had a significantly higher expression of this gene compared with birds challenged with NE18 strain. A negative correlation ($r = -0.564$, $P < 0.001$) between intestinal *OCLN* expression and blood FITC-d concentrations is shown in Figure 4.

Downregulation of IgM and IgG, MUC2 and Upregulation of MUC5ac by NE Challenge

The mRNA expression of 2 immunoglobulin genes and 2 mucin genes was also investigated in response to antibiotics and NE challenge is shown in Figure 5. Downregulation of *IgG* and *MUC2* ($P < 0.001$) and *IgM* ($P < 0.05$) were observed in both challenged groups compared with the nonchallenged birds. An antibiotic × challenge interaction was observed for *MUC5ac*, where the expression of *MUC5ac* was only upregulated ($P < 0.05$) in the NE18 group. Furthermore, an antibiotic × challenge interaction was observed for *IgM*, where the supplementation of antibiotics, only reduced the expression of this gene in the nonchallenged birds ($P < 0.05$).

Expression of Genes Encoding Digestive Enzymes and Nutrient Transporters

Nutrient transporter gene expression is shown in Figure 6. The genes *PepT2* ($P < 0.01$) and $y^{+}LAT2$ ($P < 0.05$) were downregulated in both NE challenge groups. Interestingly, $y^{+}LAT1$ gene was downregulated by the NE18 challenge ($P < 0.001$) relative to both nonchallenged and NE36 challenged treatments. An antibiotic × challenge interaction was observed in the expression of $b^{0,+}AT$ ($P < 0.05$) and $B^{0}AT$ ($P < 0.05$). The $b^{o,+}AT$ gene was downregulated by antibiotic

Table 3. Effect of necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36), on broiler chicken gut permeability and jejunal histomorphological parameters at day 16.

Main effects	FITC-d	Intestinal histology		
		Height	Crypt	Height/crypt
Antibiotic[1]				
No	0.353^{a}	453	191	3.13
Yes	0.278^{b}	472	173	2.29
Challenge				
Non	0.199^{b}	754^{a}	121^{b}	6.28^{a}
NE18	0.350^{a}	325^{b}	196^{a}	1.70^{b}
NE36	0.406^{a}	310^{b}	228^{a}	1.41^{b}
P-value				
Antibiotic[1]	0.013	0.169	0.256	0.894
Challenge	0.001	0.001	0.001	0.001
Antibiotic × challenge	0.581	0.432	0.338	0.969

[a,b]Means with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

Abbreviations: FITC-d, fluorescein isothiocyanate-dextran (ug/mL); Non, non-challenged; NE18, *C. perfringens* strains ERE-NE18, 10^{8} CFU/mL; NE36, *C. perfringens* strain WER-NE36, 10^{8} CFU/mL.

[1]Antibiotic: salinomycin (72 ppm) and zinc bacitracin (50 ppm).

treatment in only the nonchallenged birds. Challenge with NE18 and NE36 strains, however, indiscriminately downregulated $b^{0,+}AT$ gene regardless of antibiotic treatments, but the extent was different with greater downregulation in the nonantibiotic group than the antibiotic supplemented group. The gene did not show differential expression in response to the NE18 or NE36 challenge. By contrast, antibiotics upregulated the gene $B^{0}AT$ in only the nonchallenged birds, but no changes due to antibiotics were observed in challenged groups. Challenge, however, indiscriminately downregulated $B^{0}AT$ in both groups with or without antibiotic treatments to a greater extent in birds with antibiotic treatment compared with those without. On the other hand, $B^{0}AT$ gene did not show differential expression in both groups of challenged birds.

Figure 7 illustrates that the expression of *APN*, *ATP1A1*, *GLUT*, and *SI* ($P < 0.001$) were downregulated in both challenge groups. Furthermore, *GLUT2* expression was lower ($P < 0.001$) in the NE36 group compared with the NE18 group. Antibiotics upregulated the gene *APN* ($P < 0.05$) in all the treatments. In

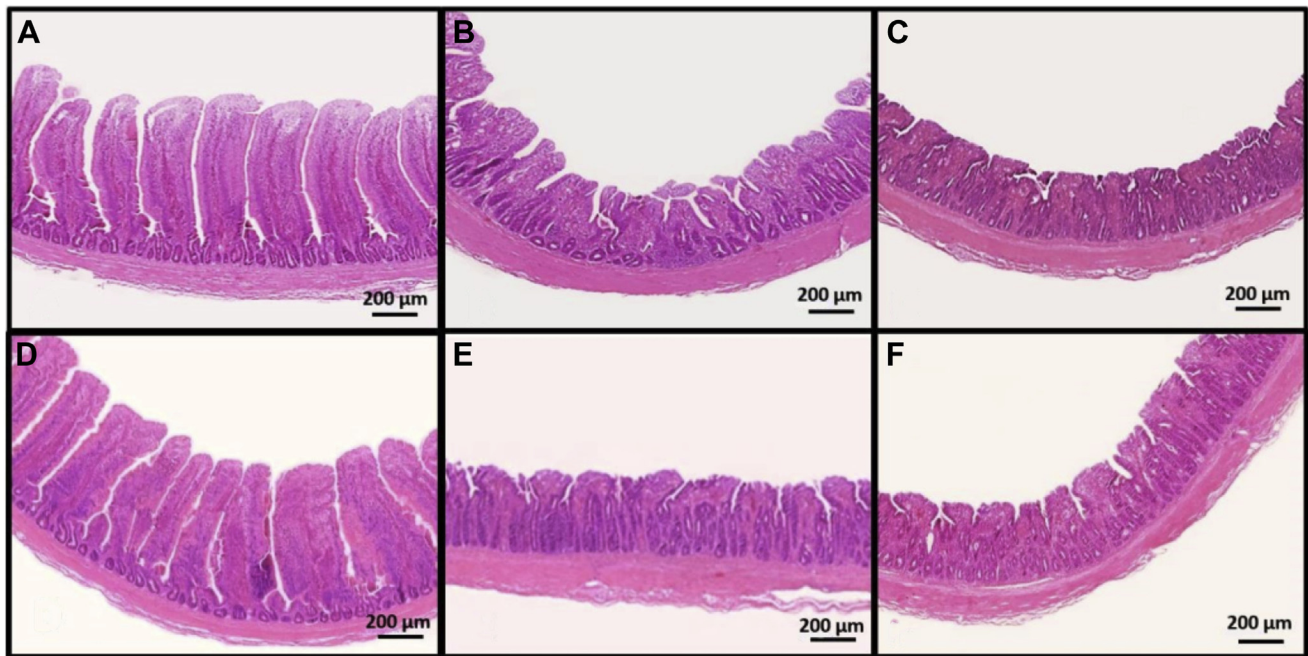

Figure 1. Effect of 2 different strains of *Clostridium perfringens* (NE18 and NE36) in a necrotic enteritis (NE) challenge model on jejunum histology at day 16. (A) Nonchallenged—no antibiotic; (B) NE challenged using NE18 strain—no antibiotic; (C) NE challenge using NE36 strain—no antibiotic; (D). Nonchallenged plus in feed antibiotics (salinomycin [72 ppm] and zinc bacitracin [50 ppm]); (E) NE challenged using NE18 strain plus in feed antibiotics (salinomycin [72 ppm] and zinc bacitracin [50 ppm]); and (F) NE challenge using NE36 strain plus in feed antibiotics (salinomycin [72 ppm] and zinc bacitracin [50 ppm]).

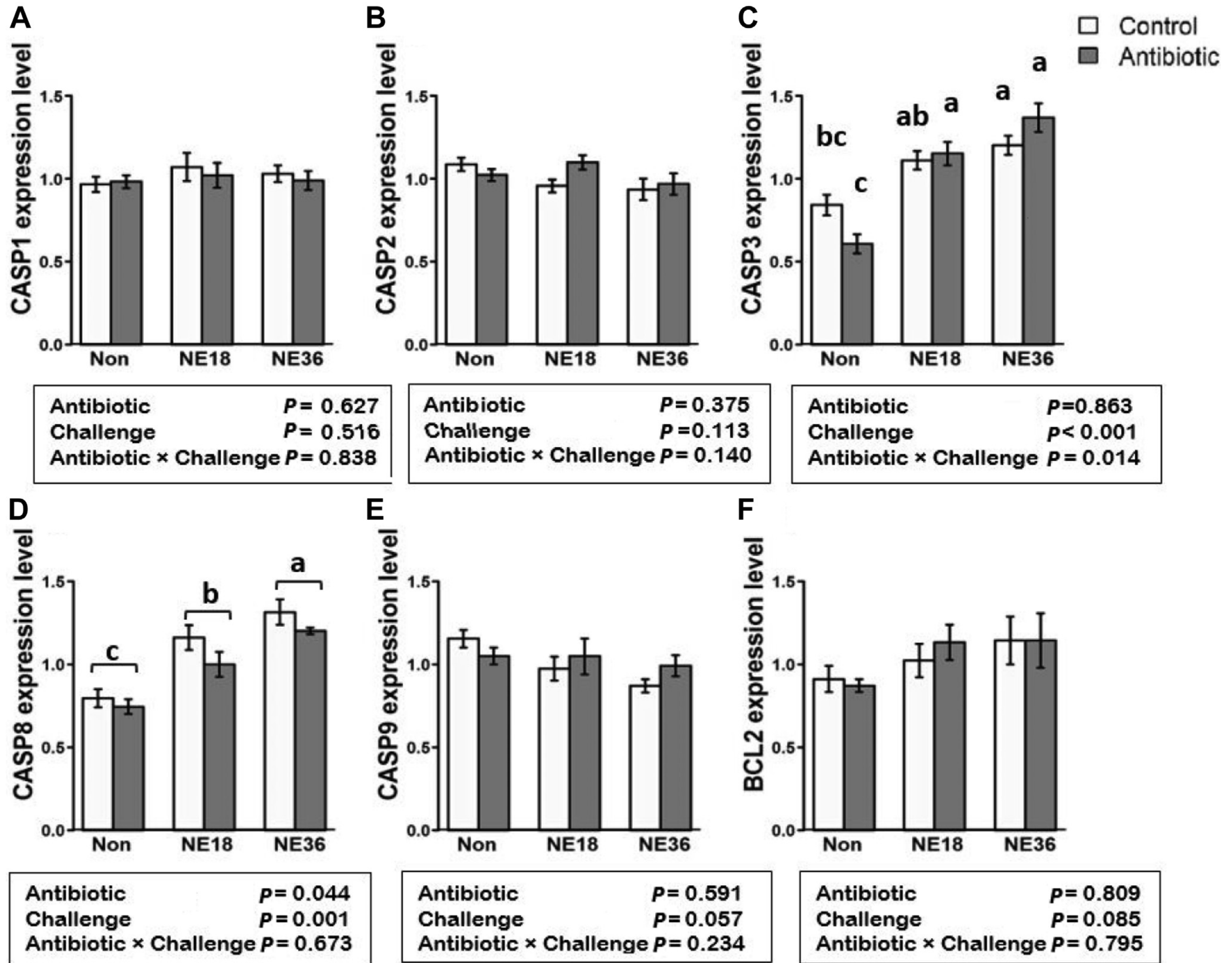

Figure 2. mRNA expression of apoptosis related genes in the jejunum tissue of broiler chickens under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36). (A) *CASP1*, B) *CASP2*, (C) expression of *CASP3* is reduced by antibiotics only in non-challenged birds. Both challenge groups increased the relative expression of *CASP3*, (D) increased relative expression of *CASP8* in both NE challenged groups. The NE36 challenge showed higher expression than the NE18. Antibiotics reduced the expression of this gene in all groups. (E) *CASP9*, (F) *BCL2*. Control: no in-feed antibiotics; Antibiotics: in-fed antibiotics (salinomycin (72 ppm) and zinc bacitracin (50 ppm)); Non: Nonchallenge. [a–c] bars with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

addition, an antibiotic × challenge interaction was observed in the expression of *ASCT1* ($P < 0.05$), where antibiotic supplementation upregulated the expression of this gene in both challenged groups, whereas no changes of *ASCT1* expression were observed among the birds without antibiotic treatment. On the other hand, this gene did not show differential expression in response to either of the *C. perfringens* strains (NE18 and NE36).

Table 4 illustrates the correlation of jejunum enzyme and nutrient transporters expression levels with broiler weight gain from day 0 to 16. Except for *BCL2* and y^+LAT1, all genes show significant correlations with weight gain. Very strong positive correlations were observed between weight gain and *GLUT2*, *APN*, *ATP1A1*, or *SI* (r = > 0.8, $P < 0.001$) whereas the strongest negative correlation was seen between weight gain and *LAT1* (r = −0.617, $P < 0.001$).

DISCUSSION

This study investigated responses of NE challenged broilers to antibiotics and 2 *C. perfringens* strains, NE18 and NE36, and compared their responses to their nonchallenged counterparts and among the challenged birds. A subclinical NE infection is characterized by significant deterioration of bird performance, mild intestinal lesions and no NE-related mortality (Skinner et al., 2010). In the present study, a successful subclinical NE infection was introduced in both challenged groups, as typical signs such as impaired FCR and BW were observed in the challenged birds (Jayaraman et al., 2013; M'Sadeq et al., 2015). Furthermore, we have previously reported higher intestinal lesion score and no significant NE-related mortality in the challenged birds (Gharib-Naseri et al., 2019) which altogether confirm a subclinical NE infection. In the present study, it was

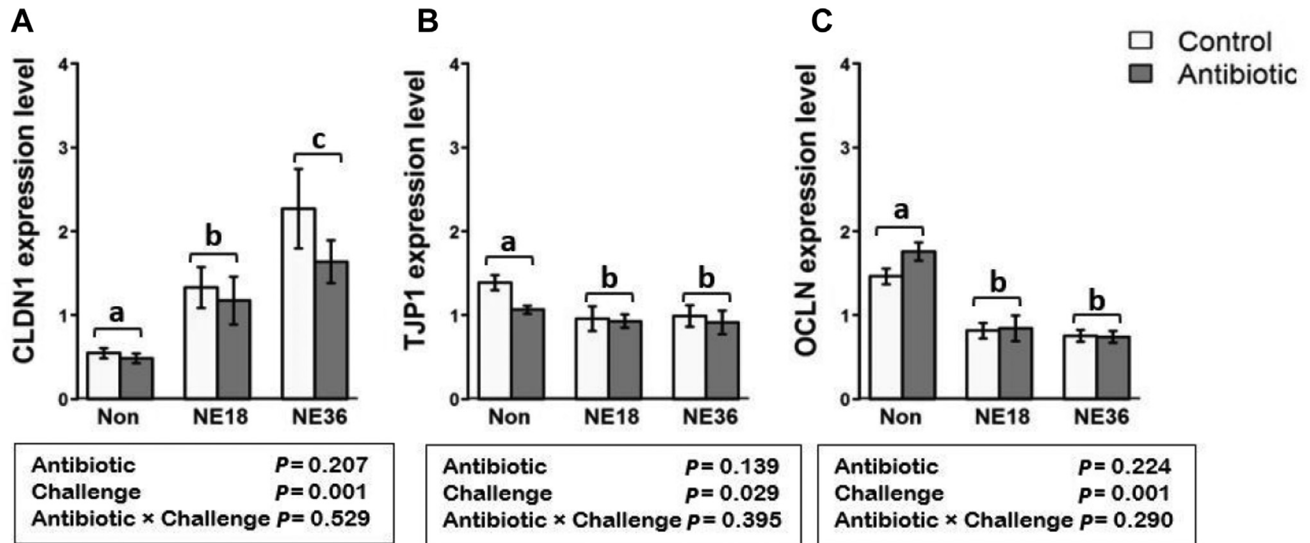

Figure 3. Relative mRNA expression of tight junction proteins in the jejunum tissue of broilers under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36). (A) Increased expression of *CLDN1* in both NE challenge groups, (B, C) downregulation of *TJP1* and *OCLN* in both NE challenge groups. Control: no in-feed antibiotics; Antibiotics: in-fed antibiotics (salinomycin (72 ppm) and zinc bacitracin (50 ppm)); Non: Nonchallenge. [a–c]bars with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

observed that, as expected, in-feed antibiotic application alleviated the negative effects of the NE challenge, and the challenge produced by either NE18 and NE36 *C. perfringens* strain introduced different levels of severity possibly through different effects on intestinal health. The study suggests that the different regulation of genes encoding TJ proteins (*CLDN1*), apoptosis (*CASP8*), mucin production (*MUC5ac*), and intestinal nutrient transporters (*GLUT2*) compared in the 2 challenge groups may underlie the mechanisms for the severity of infection in birds challenged with NE. The results of this study can lead to the acceptance of our hypothesis that NE challenge can negatively affect chicken performance through compromised intestinal gut integrity, damaged morphology, and regulation of related genes. All these may underlie the mechanism of NE infection produced by the causative agent *C. perfringens*, together with the predisposing factor *Eimeria* species applied in

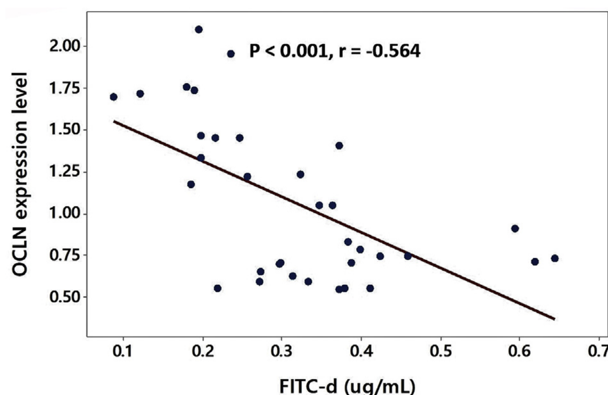

Figure 4. Correlation between FITC-d concentration in serum and *OCLN* expression in jejunum tissue of broilers chickens. Abbreviations: FITC-d, fluorescein isothiocyanate-dextran; *OCLN*, occludin.

the current challenge model. The study also suggest that the strain of *C. perfringens* with different virulence plays an important role in the severity of NE infection possibly through the modulation of intestinal gene expression responsible for immunity, intestinal integrity, mucus production, apoptosis, and nutrient transporters in the intestine.

The healthy intestinal tract is not only essential for nutrient absorption but also acts as an important barrier against bacterial infection (Balda and Matter, 2008). Therefore, intestinal integrity devised by the TJ are the leading indicators for intestinal epithelium health. Owing to large size of the FITC-d molecules (3–5 kDa), higher concentrations of this substance in the bloodstream indicates damage in the paracellular barrier, caused by *Eimeria* and *C. perfringens* challenge (Park et al., 2008b; Latorre et al., 2018). Furthermore, the reduced expression of *OCLN* and *TJP1* in the challenged birds also clearly demonstrates compromised intestinal epithelial TJ by the challenges applied. The strong negative correlation between *OCLN* expression and FITC-d concentration in the bloodstream is in agreement with the report by Cani et al. (2009) where a negative correlation between *OCLN* expression and blood FITC-d in mice suffering from inflammation disorders was observed.

Interestingly, *CLDN1* is known to be an important protein in the TJ complex, the observation in the present study showed that the significantly increased expression of *CLDN1* in challenged groups compared with nonchallenged birds appears opposite to the changes of other TJ proteins. As a transmembrane protein, *CLDN1* is a key pore-sealing TJ protein of intestinal epithelium, and it is believed that it may act differently in activated cytokinin and inflammatory situations (Pope et al., 2014). It was believed that *CLDN1* has a high affinity to

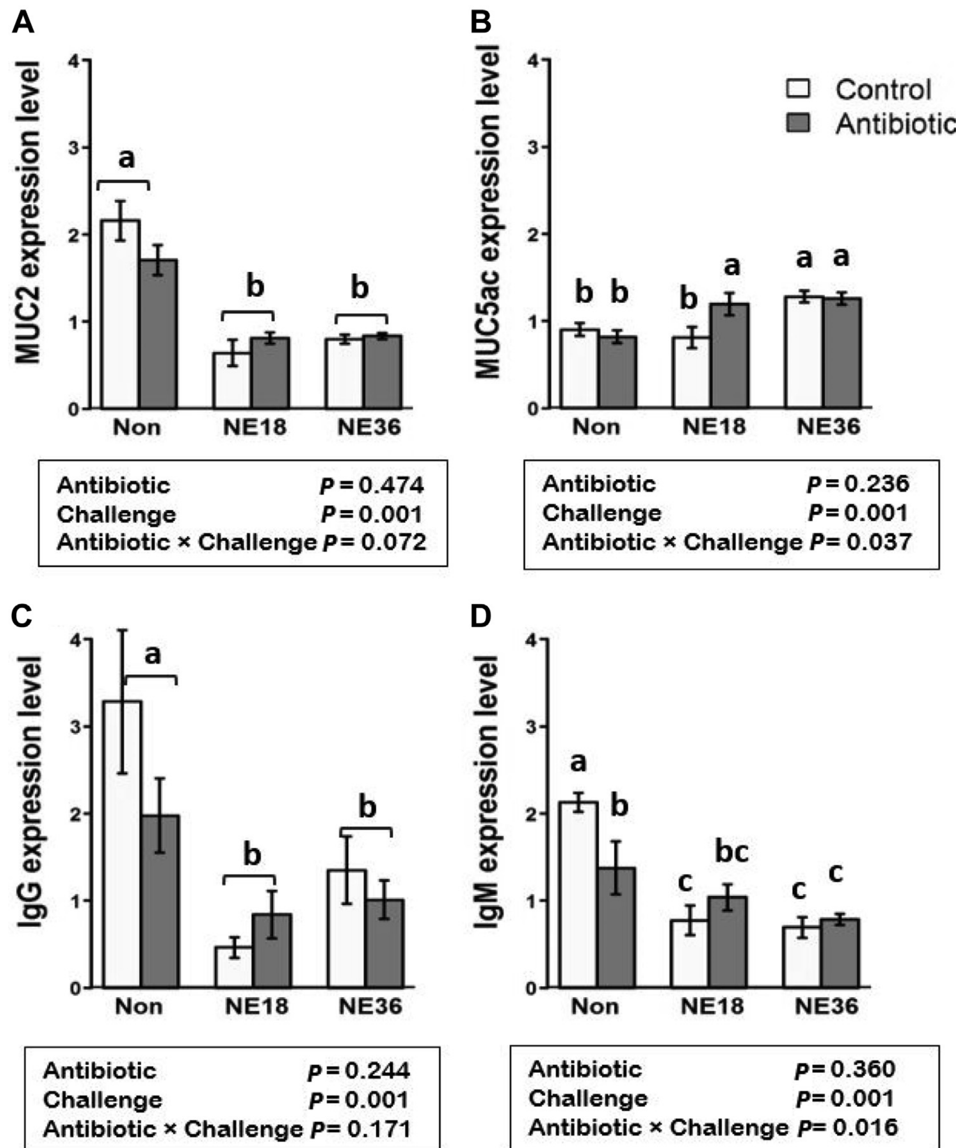

Figure 5. Relative mRNA expression of mucin- and immunoglobulin-related genes in the jejunum tissue of broilers under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36). (A) Both challenged groups downregulated expression of *MUC2*, (B) expression of *MUC5ac* was upregulated only in the NE18 challenged birds fed with antibiotic supplementation, (C) both challenged groups downregulated expression of *IgG*, (D) supplementation of antibiotics only reduced the expression of this gene in the nonchallenged birds. Control: no in-feed antibiotics; Antibiotics: in-feed antibiotics (salinomycin (72 ppm) and zinc bacitracin (50 ppm)); Non: Nonchallenge. [a–c]bars with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

C-terminal domain of *C. perfringens* enterotoxin (CPE) (Eichner et al., 2017), and the binding of CPE with *CLDN1* could form a membrane pore and lead to a massive influx of Ca^{++}, so that necrotic cell death occurs. However, another study reported that because it lacks the ECL-2 sequence favorable for CPE binding *CLDN1* is not a receptor for this toxin (Shrestha et al., 2016). Nevertheless, the NE producing *C. perfringens* strains positive in NetB toxin do not produce CPE (Rood et al., 2018), thus no CPE binding to *CLDN1* can be postulated herein. The increased expression of *CLDN1* in NE infections has been observed in the present study and reported by other researchers. Bortoluzzi et al. (2019) reported increased expression of *CLDN1* in intestinal epithelium of NE challenged broilers. Similar results were also observed by *C. perfringens* infections alone in chicken intestine (Liu et al., 2012). In humans, on the other hand, it has been reported that *CLDN1* is upregulated under disease conditions such as ulcerative colitis and active inflammatory bowel disease (Weber et al., 2008; Devriese et al., 2017; Garcia-Hernandez et al., 2017) and tumor proliferation (Huang et al., 2015; Jian et al., 2015). In a human epithelial cell investigation, Poritz et al. (2011) reported that treating the cells with different doses of tumor necrosis factor alpha caused a significant increase in *CLDN1* expression.

As suggested by Singh et al. (2010), *CLDN1* could be delocalized from the membrane to the cytoplasm and nucleus where its role becomes signal transduction as opposed to cell adhesion when it is in the membrane. It may be considered that the delocalization of *CLDN1*

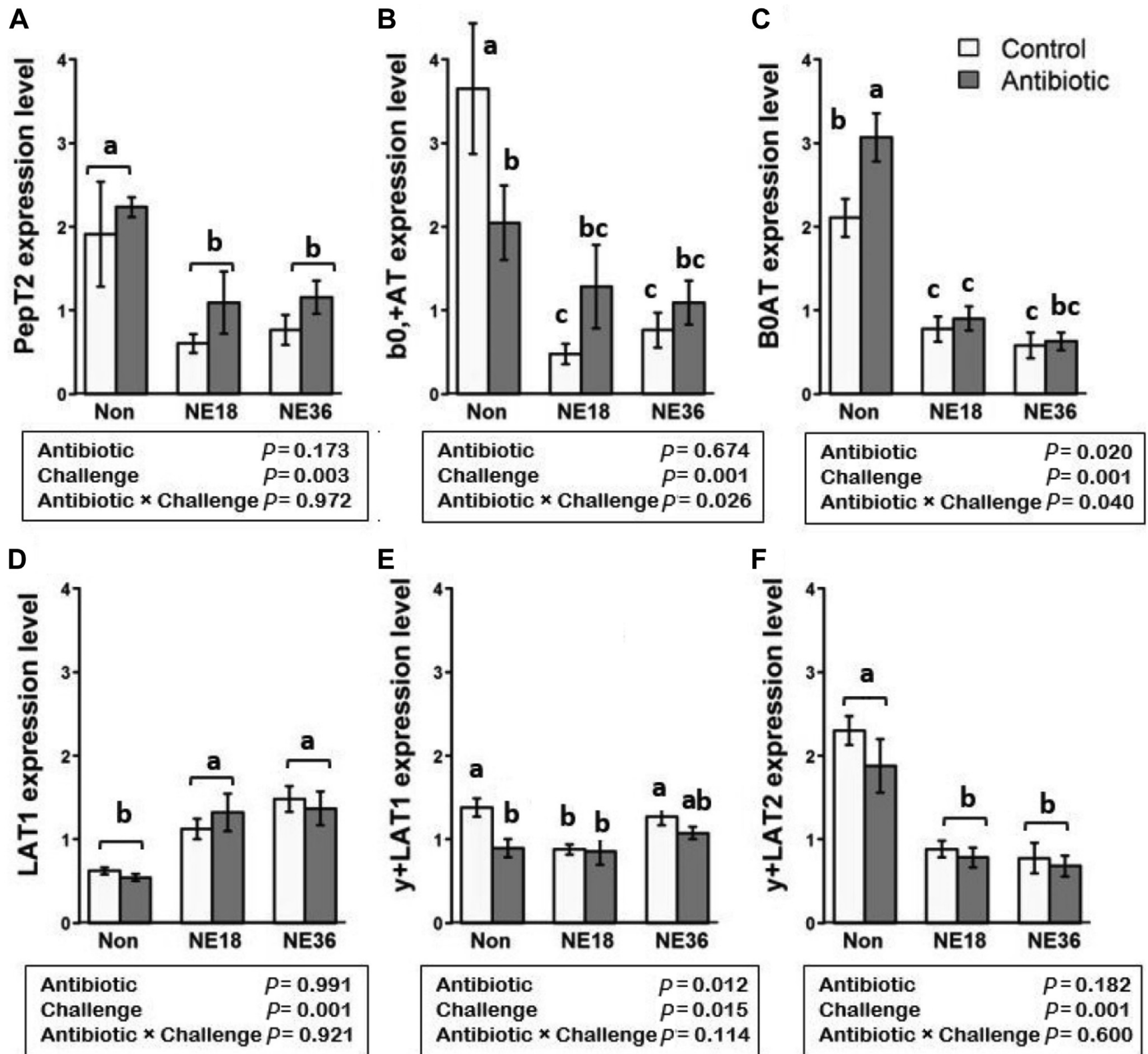

Figure 6. Relative mRNA expression of nutrient transporter genes in jejunum tissue of broilers under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36). (A) both challenged groups' downregulated expression of *PepT2*, (B) $b^{o,+}AT$ gene was downregulated by antibiotic treatment in only the non-challenged birds, (C) antibiotic supplementation upregulated $B^{0}AT$ gene in only the non-challenged birds, (D) Both challenged groups' upregulated expression of *LAT2*, (E) the NE18 challenge reduced expression of $y^{+}LAT1$, (F) both challenged groups' downregulated expression of $y^{+}LAT2$. Control: no in-feed antibiotics; Antibiotics: in-fed antibiotics (salinomycin (72 ppm) and zinc bacitracin (50 ppm)); Non: Non-challenge. [a–c]bars with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

from the membrane to cytoplasm may result in a feedback mechanism in the cell to produce more *CLDN1* so that the *C. perfringens* challenge causes increased expression of *CLDN1* as observed in the current study. It seems likely that inflammation can induce the expression of *CLDN1*. Furthermore, *CLDN1* has shown to have antiapoptotic effects in humans (Akasaka et al., 2010) and upregulation of this gene might have a protective impact in the challenged birds with increased apoptosis. Further work should be carried out to investigate whether there are any other possibilities that claudin proteins including *CLDN1* are modulated by the *C. perfringens* infection of chickens, and if so, what function does the protein have under challenge conditions. Overall, the upregulation of *CLDN1* by *C. perfringens* challenge is not necessarily an indication of TJ enhancement as may be intuitively suggested, but it is possible that a disease status of the animals is implied through its function other than as a TJ protein.

Under subclinical necrotic enteritis, intestinal epithelial cells are in a constant mode of inflammation and recovery (Star et al., 2009). This leads to shortened villus and deeper crypts which is the response of intestinal tissue to the inflammation caused by pathogens and their toxins that results in poor nutrient absorption and performance (Xu et al., 2003). In accordance with

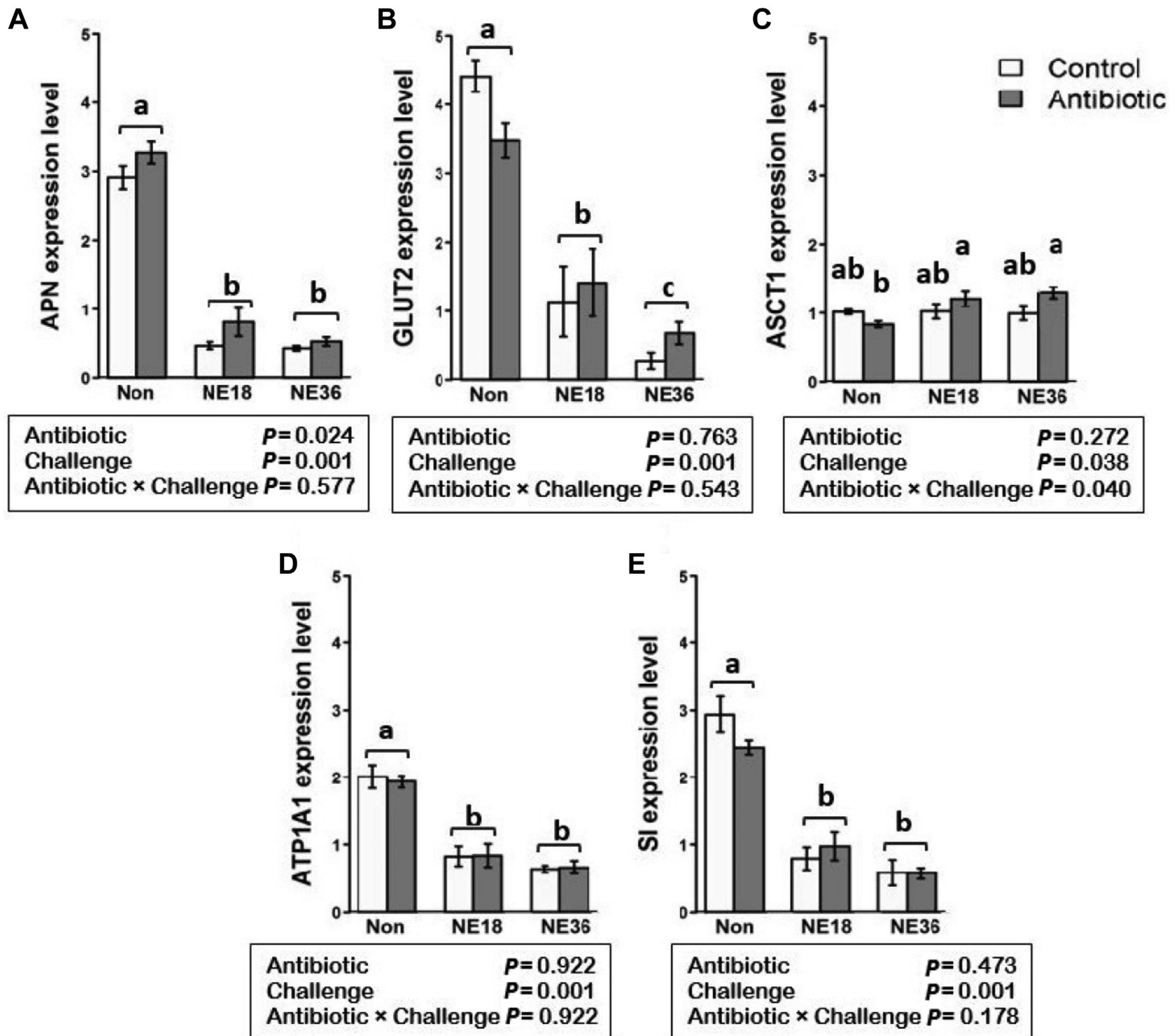

Figure 7. Relative mRNA expression of nutrient transporter genes in the jejunum tissue of broilers under necrotic enteritis (NE) challenge using 2 different strains of *Clostridium perfringens* (NE18 and NE36). (A) Both challenged groups' downregulated expression of *APN*. (B) Both challenged groups downregulated expression *GLUT2* expression and NE36 group show lower expression of this gene compared with NE18 group, (C) antibiotic supplementation upregulated the expression of *ASCT1* gene in both challenged groups, (D-E) both challenged groups' show downregulated expression of *ATP1A1* and *SI*. Control: no in-feed antibiotics; Antibiotics: in-fed antibiotics (salinomycin (72 ppm) and zinc bacitracin (50 ppm)); Non: Non-challenge. [a–c]bars with different letters significantly differ on the basis on Tukeys' multiple tests ($P < 0.05$).

histological examinations, both NE challenged groups damaged the villus structure, and these results are in agreement with other studies where subclinical NE challenge severely damaged the villis and lamina propria integrity in chicken (Timbermont et al., 2011; Wang et al., 2017). Furthermore, intestinal inflammation can

Table 4. Correlation between the expression levels of enzyme and nutrient transporter genes and weight gain of broilers challenged with 2 models of NE challenge (NE18 and NE36) during day 0–16.

	GLUT2	*APN*	*ACST1*	*BCL2*	$b^{0,+}AT$	B^0AT	*LAT1*	*ATP1A1*	*PepT2*	*SI*	y^+LAT1	y^+LAT2
WG	0.844***	0.805***	−0.336*	−0.278	0.644***	0.644***	−0.617***	0.827***	0.478**	0.865***	0.062	0.783***

Abbreviations: *APN*, aminopeptidase; *ASCT1*, alanine, serine, cysteine and threonine transporter-1; *ATP1A1*, ATPase Na+/K+ transporting subunit alpha-1; *BCL2*, B-cell lymphoma-2; $b^{0,}+AT$, $b^{0,+}$amino acid transporter; B^0AT, neutral amino acid transporter; *GLUT2*, glucose transporter-2; *LAT1*, large neutral amino acid transporter-1; *PepT2*, peptide transporter-2; *SI*, sucrase-isomaltase; WG, weight gain; y^+LAT1, Y + L amino acid transporter-1; y^+LAT2, Y + L amino acid transporter-2; WG, weight gain.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

decrease goblet cell numbers, which are the primary sites for mucin gel formation in the intestine (Freitas et al., 2002; Tan et al., 2014; Wu et al., 2018). *MUC2* synthesis takes place in the goblet cells, and deficiency of this protein has shown to increase bacterial translocation and intestinal inflammation (Wei et al., 2012). Histological analysis of the jejunum tissue in the present study revealed that NE challenge caused sever shedding in the epithelial layer. It could be then suggested that the downregulation of *MUC2* is caused by the intestinal mucosa deterioration, due to the damage caused by NE infections, preventing mucus from renewal and increasing the chance of further infection. Significant damage to the gut structure caused by the NE challenge can also reduce nutrient uptake, which in turn lower immunoglobulin responses, as shown by Konashi et al. (2000). A recent subclinical necrotic enteritis study reported a significant decrease of IgA + B cells in infected chickens (Wang et al., 2017) that supports the findings observed in this study.

Interestingly, *MUC5ac* was upregulated by the NE36 challenge. In humans, Park et al. (2008a) demonstrated an increase of *MUC5ac* in patients with colon cancer and Forgue-Lafitte et al. (2007) observed such an increase in patients with ulcerative colitis. This phenomenon was also observed in NE (Forder et al., 2012) and *Eimeria*-infected (Kitessa et al., 2014) broiler chickens previously. Unlike *MUC2* which is widely expressed in the intestine, *MUC5ac* is highly expressed in the stomach (Van Klinken et al., 1995), and the role of *MUC5ac* might be different from *MUC2* in the intestine thus this may cause a different response to disease challenge. Cell damage and lysis caused by *Eimeria* sporozoites penetration in epithelium cells and toxins produced by *C. perfringens* can generate signals that initiate conformational changes in apoptosis proteins (Gao and Kwaik, 2000). Caspases (cysteine-aspartic proteases, cysteine aspartates, or cysteine-dependent aspartate-directed proteases) play a critical role in regulating programmed cell death, including apoptosis, and inflammation (McIlwain et al., 2013). Extrinsic and intrinsic pathways are the 2 main pathways that mediate cell death. The extrinsic pathway is activated by *CASP8* which activates executioner caspases (i.e., *CASP3*, −6, and −7) in their inactive form, that is, procaspase dimer state (Nunes et al., 2005; McIlwain et al., 2013). Caspase-3 is critical for the execution of the apoptotic process in both pathways (Riedl and Shi, 2004). In the present study, elevated *CASP8* and *CASP3* expression by the challenge suggests an extrinsic activated cell death pathway in the intestine of challenged birds.

Amino acids and energy uptake are also important factors affecting the immune functions and susceptibility to disease in animals (Broer, 2008). Nutrient uptake is mostly regulated by specific transporters across the plasma membrane in the small intestine, and changes in these nutrient transporters may underlie reduced body weight and feed efficiency in NE challenged birds. Na^+-dependent neutral/cationic amino acid exchanger such as light chain heteromeric amino acid transporters (*LAT1*, y^+LAT1, y^+LAT2), Na^+-dependent neutral amino acid transporters such as $b^{0,+}AT$, B^0AT, and *ASCT1*, and glucose transporters (*GLUT1-2*) are all the proteins transporting respective nutrients thus their uptake in the epithelium layer. Moreover, most immune responses to pathogens are characterized in decreased appetite and in directing nutrients away from skeletal muscle accretion toward hepatic production and secretion of acute-phase proteins (Humphrey and Klasing, 2004). The addition of antibiotics showed a significant improvement in the performance and gut integrity of challenged birds, and upregulated the expression of *ASCT1* in both NE-infected groups. Nonetheless, no other significant effect of antibiotics was observed in the challenged birds. We speculate that the immune system or changes in the microbiota population could have affected the expression of the genes in response to the antibiotic supplementation. Further investigation is needed to understand the relation between the effect of antibiotics and the expression of intestinal genes in NE challenge broilers.

The *SI* gene is usually found on the surface of intestinal epithelial cells, where it enables the production of the sucrase-isomaltase enzyme and is the key to starch and sugar degradation. This enzyme breaks sucrose and maltose into simple sugars to be absorbed in the intestinal epithelial cells (Diaz-Sotomayor et al., 2013). *GLUT2* is present on both basolateral and apical membrane of the intestine, facilitating glucose absorption. It transports monosaccharides from enterocytes into the blood (Kellett et al., 2008). Downregulation of *GLUT2* and *SI* can lead to diminished transport of carbohydrate to the tissues and result in reduced weight gain of the broilers (Su et al., 2014), such as in the case observed in the NE36 group of this study. Aminopeptides such as APN are highly expressed on the brush border of the epithelium and are responsible for final digestion of peptides by N terminus cleavage (Gal-Garber and Uni, 2000). In both challenged groups (NE18 and NE36), the expression of *APN* was decreased, which may cause reduced nutrient absorption and negatively affect the gut barrier integrity and immune responses (Luan and Xu, 2007). The strong correlations between these genes and the weight gain of chickens may suggest that downregulation of these genes can, at least partially, be responsible for the depleted growth of challenged birds. Furthermore, the downregulation of brush-border transporters, such as $b^{\circ,+}AT$ and B^0AT that regulate free amino acid uptake to the epithelial cells, initiated by the challenge may also show the adverse effects of this infection on the growth and feed efficiency of birds probably by the depleted influx of essential amino acids into epithelial cells which are critical for absorption.

CONCLUSIONS

Taken together, our results suggest that both NE challenges produced by the 2 *C. perfringens* strains, compromised performance, gut integrity and intestinal

morphology. The positive effect of antibiotics was evident on the birds; however, owing to the more severe impact of the NE36 challenge on the bird's performance, the effectiveness of antibiotics was lower compared with the NE18 challenge. Further differences were observed in the expression of genes related to TJ, cell death, mucin production, and intestinal transporters between the 2 challenged groups. The mode of action on how *C. perfringens* produce clinical or subclinical NE has been elucidated as the action of causative toxin NetB (Keyburn et al., 2008). However, the mechanism underlying how these two NE challenges affect the severity of the disease is yet to be illustrated. The differences observed in the expression of intestinal genes in the birds challenged with NE18 and NE36 may provide preliminary evidences on how the hosts respond to the challenge. Further investigation on the genomic factors in the bacteria contributing to these different responses would be appealing for more in-depth study of *C. perfringens* virulence that leads to different levels of severity of the NE disease.

ACKNOWLEDGMENTS

The authors thank Ms. Petrina Young of Eimeria Pty Ltd. for providing *Eimeria* species. Prof. Robert Moore for providing *Clostridium perfringens* EHE-NE18 and WER-NE36, and Mr. Jonathan Clay for his help and guidance with the laboratory work.

DISCLOSURES

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence the work; there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the content of this article.

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