

Differential gene expression profiles of β -defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines

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ABSTRACT Changes in the expression levels of avian β -defensin (*AvBD*) mRNA were evaluated in necrotic enteritis (NE) disease model in 2 genetically disparate commercial broiler chicken lines: Ross and Cobb. The NE was initiated in the gut by a previously established co-infection model using oral *Eimeria maxima* infection followed by a *Clostridium perfringens* challenge. Among the 14 *AvBD* types examined, there was a tissue-specific expression of *AvBD* transcripts: *AvBD1*, *AvBD7*, and *AvBD9* in the crop; *AvBD8*, *AvBD10*, and *AvBD13*; in the intestine and *AvBD1* and *AvBD7* in the spleen. The 2 different commercial broiler chicken lines showed differential gene expression patterns of *AvBD* transcripts following co-infection with *E. maxima* and *C. perfringens*, with R-line chickens generally showing

higher expression levels than the C strain. Both chicken strains showed enhanced gene expression levels of proinflammatory cytokines, such as *IL-1 β* , *IL-6*, *IL-17F*, and *TNFSF15* in spleen, and *TNFSF15* in intestine, whereas *IL-17F* was significantly increased only in the intestine of R-line chickens following NE infection. Although the exact nature of interactions between defensins and cytokines in determining the outcome of host innate immune responses to the pathogens of NE remains to be investigated, the differences in gene expression levels of β -defensins and proinflammatory cytokines in the intestine, crop, and spleen could explain the predisposed disease resistance and susceptibility to NE in the 2 commercial broiler chicken lines.

Key words: chicken, β -defensin, cytokine, *Eimeria maxima*, *Clostridium perfringens*

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INTRODUCTION

In the United States, necrotic enteritis (NE) is among the most important infectious diseases in chickens (Smith and Helm, 2008). Globally, the economic loss due to NE is estimated to cost the US \$2 billion annually, largely because of medical treatments and impaired growth performance (Van Immerseel et al., 2009). Recently, NE has reemerged as a significant problem as a result of restricted use of in-feed antibiotics, high-density housing conditions, and reuse of litter. Thus, there is an urgent need to develop rational and alternative β -defensin management strategies not only to control but also to prevent NE (Williams, 2005). Better understanding of host-pathogen, as well as pathogen-pathogen (*Clostridium-Eimeria*), interactions in NE will be required to realize these goals.

Necrotic enteritis is difficult to experimentally reproduce with *Clostridium perfringens* alone, and a variety of factors have been identified that promote the development of NE (Park et al., 2008; Lee et al., 2011). *Eimeria maxima* is the most common co-infection species used in experimental NE in *C. perfringens*-infected birds (Williams et al., 2003; Park et al., 2008; Miller et al., 2010). Necrotic enteritis infection causes many changes in immunological parameters, including cytokines and toll-like receptors (Collier et al., 2008; Park et al., 2008), but the nature of innate immune response, especially the role of defensins in NE, remains to be determined.

Defensins are antimicrobial peptides of relatively small molecules with less than 100 amino acids that contribute to the antimicrobial action of granulocytes as well as mucosal defense in the gut and epithelial surfaces. There are 2 main defensin subfamilies, α - and β -defensins, that differ in the length of peptide segments between the 6 cysteins (Ganz, 2003). The α -defensins are unique to mammals and are not expressed in birds (Lynn and Bradley, 2007), whereas β -defensins

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are found throughout vertebrate species (Lehrer and Ganz, 2002; Lynn, et al., 2007). Unlike other animals, primates also possess a third type, theta(θ)-defensin (Tang et al., 1999).

β -Defensins represent important effector molecules of host innate immunity, and they have been isolated from leukocytes and epithelial cells of skin, gastrointestinal, and respiratory tracts. Proinflammatory cytokines, such as IL-1 β , IL-6, and TNF- α are known to be potent inducers and upregulators of defensins, such as human β -defensin-2 (Hancock and Diamond, 2000; Scott and Hancock, 2000; McDermott, 2006).

Interestingly, avian species express only β -defensins which have been identified in peripheral blood of chicken, turkey, and ostrich; epithelial cells of chicken and turkey; king penguin stomach contents; king pigeon's bone marrow; spleen of mallard ducks; and Japanese quail (Sugiarto and Yu, 2004; Higgs et al., 2005; Lynn et al., 2007). In chickens, 14 β -defensin genes (*AvBD1* to 14) have been identified in the leukocytes, epithelial cells, or EST of chicken genome (Evans et al., 1994; Harwig et al., 1994; Zhao et al., 2001; Lynn et al., 2004; Xiao et al., 2004; Higgs et al., 2005; Lynn et al., 2007).

The antimicrobial activity or transcriptional profiles of β -defensins against pathogens, such as *Salmonella enterica* serovar Typhimurium, *C. perfringens*, or *Escherichia coli* have been reported using in vivo or in vitro studies. Chicken β -defensin gallinacin-6 (*AvBD9*) plays an important role in innate host defense against foodborne pathogens in chicken digestive tract (van Dijk et al., 2007), and the expression levels of *AvBD1*, *AvBD2*, *AvBD4*, and *AvBD6* (Akbari et al., 2008) as well as gallinacin 4, 7, and 9 (Milona et al., 2007) were significantly increased in cecal tonsils of young chickens following infection with *Salmonella* Typhimurium. In addition, differential transcriptional profiles of β -defensins in response to *Salmonella enteritidis* were reported in ovaries (Michailidis et al., 2012), gut (Crhanova et al., 2011), and in the intestinal epithelial cells between resistant and susceptible inbred chicken lines (Derache et al., 2009). However, there have been no studies showing the expression of β -defensins in NE in chickens yet. Therefore, we examined the expression profiles of *AvBD* transcripts in 3 different tissues to compare *AvBD* involvement in NE using 2 commercial broiler chicken strains showing disparate NE disease susceptibility.

MATERIALS AND METHODS

Birds and NE Infection

Two different commercial broiler lines, Cobb and Ross, were obtained from Mountaire Farms in Millsboro, DE. Chickens were kept in brooder pens in a disease-free facility for 14 d posthatch and provided with feed and water ad libitum. They were then transferred to large hanging cages (2 birds/cage) at a separate location where they were given an oral infection with 1.0×10^4 sporulated oocysts of *E. maxima*, followed with

C. perfringens (1.0×10^9 cfu) 4 d later. *Clostridium perfringens* were maintained and propagated using the previously described method (Lee et al., 2011). All experiments were performed according to the guidelines established by the Beltsville Agriculture Research Center Small Animal Care Committee.

Tissue Collection and cDNA Synthesis

Spleen, crop, and intestinal jejunum tissues were freshly collected from 5 chickens per group at 2 d post *C. perfringens* infection and pooled for total RNA extraction. Total RNA was extracted as described (Lee et al., 2010). Intestine and crop were cut longitudinally and briefly washed 3 times with ice-cold Hanks' balanced salt solution containing 100 U/mL of penicillin and 100 mg/mL of streptomycin (Sigma, St. Louis, MO). The mucosal or inner layer was carefully removed using a cell scraper (Nunc, Thermo Fisher Scientific Inc., Roskilde, Denmark), and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). To synthesize cDNA, 5 μ g of total RNA were treated with 1.0 U of DNase I in 1.0 μ L of $10\times$ reaction buffer (Sigma) and incubated for 15 min at room temperature. One microliter of stop solution was added to inactivate the DNase I. It was then heated at 70°C for 10 min and reverse-transcribed at 42°C for 1 h using the StrataScript first strand synthesis system (Stratagene, La Jolla, CA) with 5.0 μ g of oligo (dT) primer, 25 mM of dNTPs, and 50 U of reverse transcriptase in a total volume of 19 μ L.

Quantitative Real-Time PCR

Oligonucleotide primers for chicken β -defensin, proinflammatory cytokines, and chicken GAPDH control were designed based upon sequences available from public databases and listed in Table 1. Amplification and detection were carried out using equivalent amounts of cDNA from spleen, crop, and intestine using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described by the manufacturer (Hong et al., 2006a). Standard curves were generated using \log_{10} diluted cDNA obtained from pooled infected samples for each chicken β -defensin and non-infected total RNA. The levels of individual transcripts were normalized to those of GAPDH and gene expression was analyzed by the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression before calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression using the Q-gene program (Muller et al., 2002; Hong et al., 2006a,b).

Statistical Analysis

Mean \pm SE values for each group ($n = 5$) were calculated and differences between groups were analyzed

Table 1. Sequences of the oligonucleotide primers used in quantitative real-time PCR

RNA target	Primer sequence (5'-3')		Size for PCR product (bp)	Accession no.
	Forward	Reverse		
GAPDH	TGCTGCCCAGAACATCATCC	ACGGCAGGTCAGGTCACAAA	142	K01458
Defensin-1	GAAAACCCGGGACAGACG	AGCGAGAAGCCAGGGTGAT	265	NM_204993
Defensin-2	TCTGCAGCCATGAGGATTC	TAAAGCACATGCCTGGAAGAAAT	259	NM_204992
Defensin-3	ACCCAGTGCAGAATAAGAGGAG	AGACTGCAGAGGTGGGAAGG	204	AY677340
Defensin-4	AATCCTTTGCTTTTTTCATC	TGCAATTATTCACACTACAACCAT	201	NM_001001610
Defensin-5	GTGACCCTCCGGGCATCT	ATGAACGTGAAGGGACATCAGAG	253	NM_001001608
Defensin-6	ATCCTTTACCTGCTGCTGTCTGT	GAGGCCATTTGGTATGTC	250	NM_001001193
Defensin-7	GCTGTCTGTCTCTTTTGTGGTG	ATTTGGTAGATGCAGGAAGGAT	227	NM_001001194
Defensin-8	TGTGGCTGTTGTGTTTTFT	CTGCTTAGCTGGTCTGAGG	267	NM_001001781
Defensin-9	ACCGTCAGGCATCTTCACAG	CCATTTGCAGCATTTTCAGC	241	NM_001001611
Defensin-10	GAATTGGGGCACGCAGTC	CCGGAATCTTGGCACAGC	229	NM_001001609
Defensin-11	ACTGCATCCGTTCCAAAGTCTG	GTCCCAGCTGTTCTTCCAG	168	NM_001001779
Defensin-12	ACCTTTGTTTCGTGTTTCATCTTC	AGGTGCTGCTGCTCTCCA	230	NM_001001607
Defensin-13	CATCGTTGTCTATTCTCCTCCTC	GGTGGAGAACCTGCAGCAGCG	163	NM_001001780
Defensin-14	ATGGGCATATTCCTCTG	CTTTGCCAGTCCATTGTAG	159	AM402954
IL-1 β	TGGGCATCAAGGGCTACA	TCCGGTTGGTTGGTGATG	244	NM_204524
IL-6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTCT	254	NM_204628
IL-17F	CTCCGATCCCTTATTCTCCTC	AAGCGGTTGTGGTCTCAT	292	NM_204460
TNFSF15	CCTGAGTTATTCCAGCAACGCA	ATCCACCAGCTTGATGTCACTAAC	292	NM_001024578

by the Student's *t*-test using SPSS software (SPSS 18.0 for Windows, Chicago, IL). Statistical differences were considered significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Expression of Chicken β -Defensins in NE

The expression levels of *AvBD*, which are induced following *E. maxima* and *C. perfringens* infections in spleen, crop, and intestine, were assessed in 2 different commercial broiler lines. The crop is essentially an enlarged part of the esophagus that stores food temporarily and shows a high level of Gal-6 (*AvBD9*) expression (van Dijk et al., 2007). As shown in Figure 1, chickens with NE showed high levels of *AvBD1*, 7, and 9 and moderate expression of *AvBD2*, 6, and 11. In contrast, other β -defensin genes showed minimum (*AvBD4*, 5) or no expression.

Following *E. maxima* and *C. perfringens* co-infection, the expression of 8 out of 14 β -defensins were detected in the jejunum (Figure 2): *AvBD8*, 10, 13 with high levels of mRNA transcripts, and *AvBD1*, 6, 9, 11, 12 with moderate gene expression levels. High level expression of gallinacin 11 (*AvBD13*) in the small intestine, liver, gall bladder, and spleen in poultry has also been previously reported against the intestinal pathogens *Salmonella* Typhimurium and *Listeria monocytogenes* (Higgs et al., 2005).

Compared with crop and intestine, baseline expression levels of *AvBD1* and *AvBD7* were significantly higher in spleen, with moderate levels of expression of *AvBD2*, *AvBD4*, and *AvBD6* (Figure 3). Furthermore, the expression levels of some β -defensin genes, such as *AvBD3*, *AvBD5*, *AvBD8*, *AvBD13*, and *AvBD14* were

significantly different when comparing the uninfected control and NE-infected chickens (Figure 3).

Differential mRNA Expression of Chicken β -Defensins in 2 Lines

Two major commercial broiler chicken lines were used to investigate genetically determined differences in the expression of defensins in response to NE. As shown in Figure 1, Ross-line chickens expressed higher levels of β -defensins, when compared with Cobb chickens, regardless of the infection status in the crop. The gene expression levels of *AvBD1* and *AvBD7* were significantly different between control and NE Cobb lines, although no significant differences were observed between the uninfected control and the infected chickens in *AvBD1*, 7, and 9 of Ross chickens. This finding is consistent with the high gene expression of Gal-6 (*AvBD9*) in the esophagus and crop of broiler chicken (van Dijk et al., 2007). Interestingly, the expression levels of *AvBD2* (in Ross line), 6 (in Cobb and Ross lines), and 11 (in Cobb line) genes were significantly different within the same broiler chickens. These results indicate that the crop plays an important role in local innate host defense against enteric pathogens, which has previously been suggested by others (van Dijk et al., 2007).

In the intestine, mRNA level of *AvBD1*, 6, 8, and 10 were significantly higher in Ross compared with Cobb chickens (Figure 2). *AvBD8* was preferentially expressed in Ross chickens in response to NE, which indicates a potential protective role of this defensin in local protection against *Eimeria* and *C. perfringens* infections (Figure 2). Interestingly, the gene expression of *AvBD8* is known to be significantly increased in epithelial cells of the liver, gall bladder, spleen, and small intestine following infections with *E. coli*, *Listeria monocytogenes*,

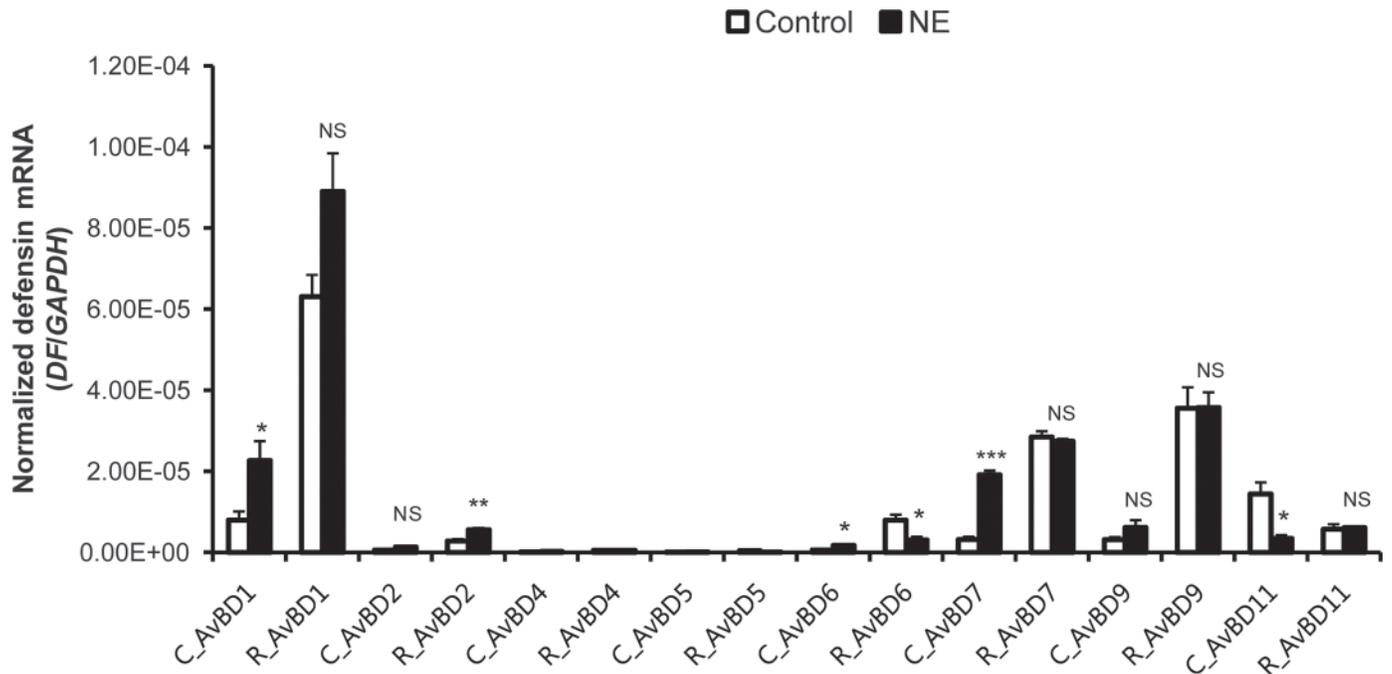


Figure 1. The β -defensin expression profiles in the crop of 2 commercial broilers [Cobb (C) vs. Ross (R)]. Chickens were noninfected or orally infected with 1.0×10^4 oocysts of *Eimeria maxima* on d 14 and following *Clostridium perfringens* infection with 1.0×10^9 cfu at 4 d later. Crops were obtained from 2 d post-*C. perfringens*-infected or noninfected chickens, and transcriptional levels were determined by quantitative real-time PCR. Data are expressed as normalized mRNA levels to *GAPDH* mRNA levels of triplicate determinations with pooled samples from 5 chickens. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant, NE = necrotic enteritis.

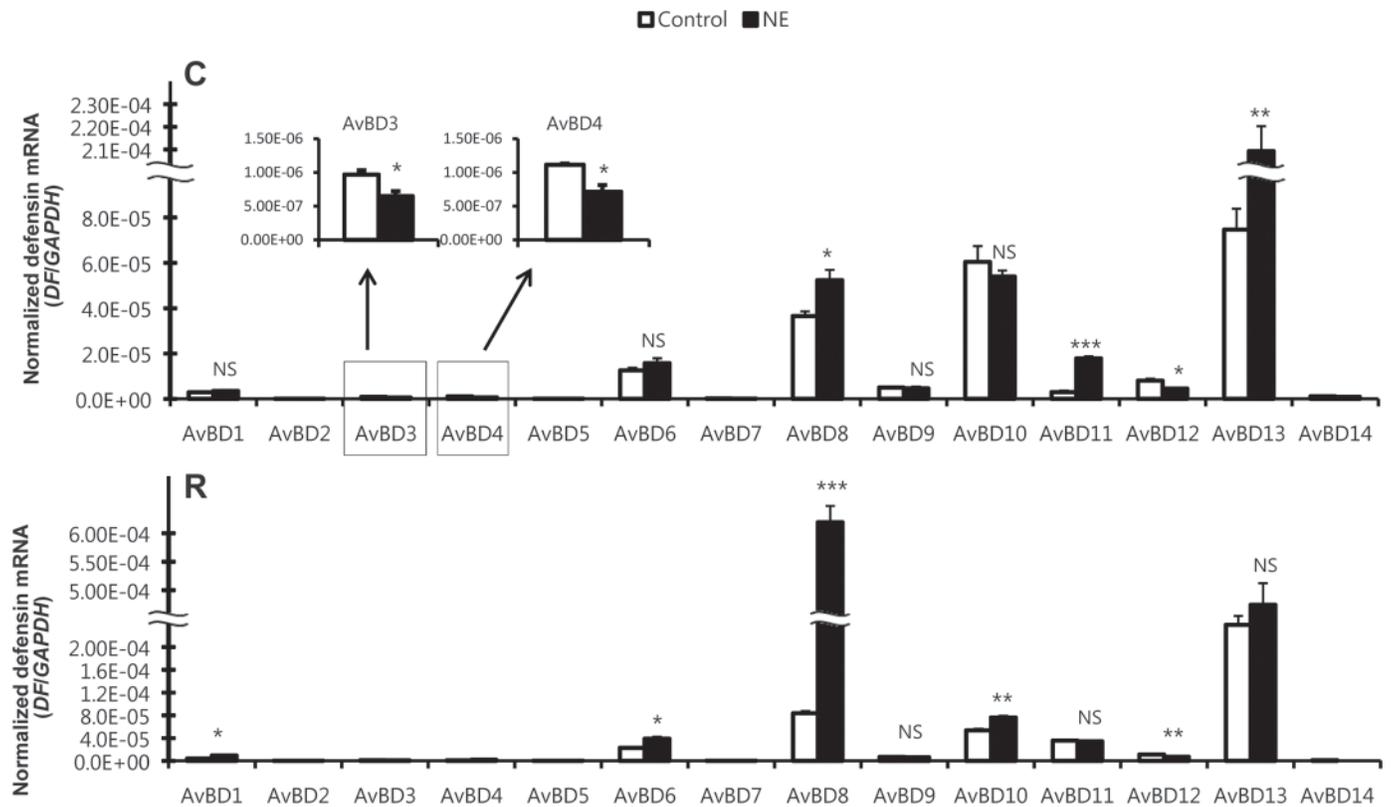


Figure 2. Chicken β -defensin expressions in the intestinal mucosal layer of 2 commercial broiler chicken lines, Cobb (C) and Ross (R). Intestinal mucosal layers were isolated from the jejunum of 2 d post-*Clostridium perfringens* infection from normal and infected chickens, and mRNA levels were determined by quantitative real-time PCR. Data are expressed as normalized mRNA levels to *GAPDH* mRNA levels of triplicate determinations with pooled samples from 5 chickens. To show a lower level of significant β -defensin expression, small graphs were inserted in the figure. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant, NE = necrotic enteritis.

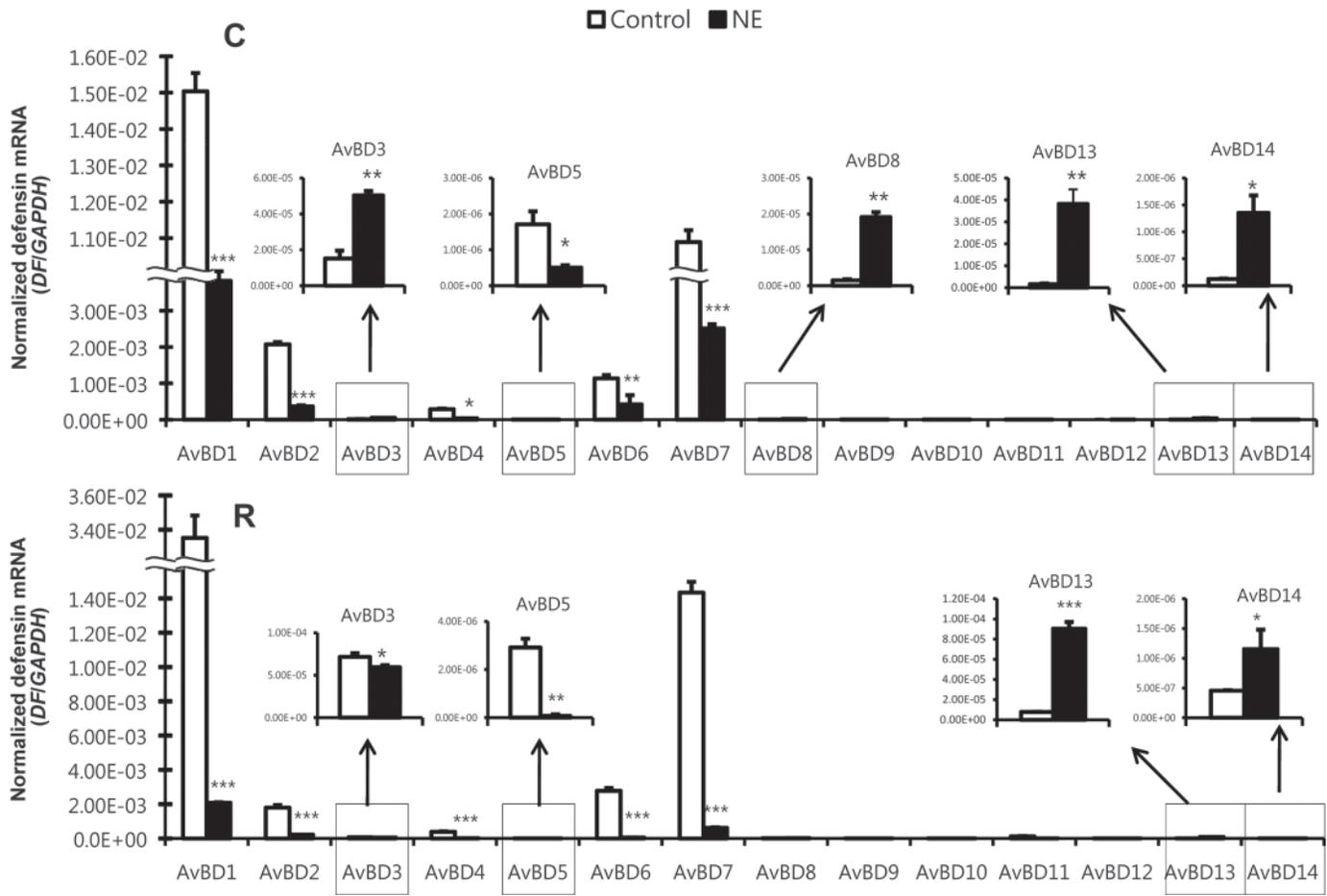


Figure 3. Chicken β -defensin expression patterns in the spleen of 2 commercial broiler breeds [Cobb (C) vs. Ross (R)]. Splens were isolated from 2 d post-*Clostridium perfringens* infection. Pooled mRNA samples from normal and *C. perfringens*-infected chickens were used in quantitative real-time PCR. Data are expressed as normalized mRNA levels to *GAPDH* mRNA levels of triplicate determinations with pooled samples from 5 chickens. To show a lower level of significant β -defensin expression, small graphs were inserted in the figure. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant, NE = necrotic enteritis.

Salmonella Typhimurium, and *Streptococcus pyogenes* (Higgs et al., 2007).

In spleen, *AvBD* gene expression patterns were quite different: β -defensins in spleen showed no increases postinfection with *E. maxima* and *C. perfringens*. In the spleen, the levels of *AvBD* genes were significantly decreased in both Ross and Cobb chicken lines (Figure 3). In addition, *AvBD3* and *AvBD8* gene expression levels were significantly increased in Cobb chickens, whereas *AvBD3* was significantly decreased in Ross with no discernible difference in *AvBD8* in Ross. Moreover, baseline mRNA transcripts of *AvBD1* and *AvBD6* in Ross chickens were 2 times higher than Cobb in uninfected groups.

Immune Response and Induction of β -Defensin Expression in Chicken Gut

Our earlier studies showed that many different types of proinflammatory cytokines are increased following infection with *Eimeria* or *C. perfringens* (Hong et al., 2006a,b; Park et al., 2008). These pathogens elicit the production of proinflammatory cytokines, such as

TNF- α , IL-1 β , and IL-6 (Sugiarto and Yu, 2004), and several β -defensins, which are known to be upregulated in inflammation (van Dijk et al., 2007). For this reason, we assessed proinflammatory cytokines in Ross and Cobb chickens.

Compared with proinflammatory cytokine expression levels of the control and NE-infected chickens in intestinal mucosal layer, *IL-1 β* and *IL-6* showed no significant differences, whereas *IL-17F* and *TNFSF15* mRNA transcriptional levels were significantly increased in NE-infected birds in both chicken lines in response to NE, except for *IL-17F* in the Cobb line (Figure 4).

In the spleen, all proinflammatory cytokines that were examined showed significant increases following NE infection, with Ross expressing higher than Cobb chickens, although *TNFSF15* was highly induced in NE-infected Cobb chickens (Figure 4).

DISCUSSION

Necrotic enteritis in broiler chickens is caused by *C. perfringens* infection following gut damage caused by infections such as coccidiosis in the midintestine.

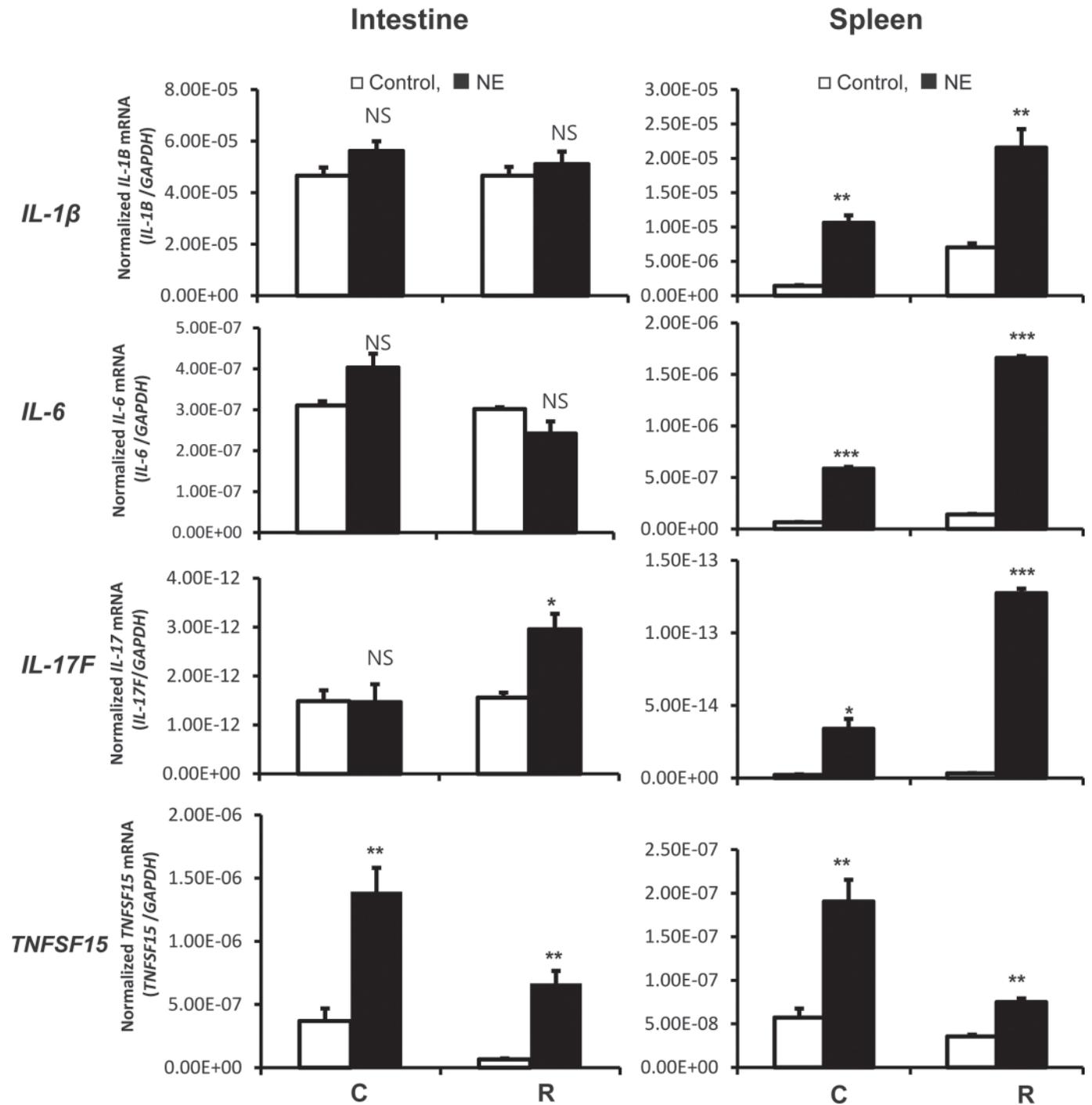


Figure 4. Proinflammatory cytokine mRNA levels in intestine and spleen of 2 commercial broiler chickens: Cobb (C) and Ross (R). Intestinal mucosal layer from jejunum and spleen were obtained at the indicated time points postinfection, and mRNA levels were determined by quantitative real-time PCR. Data are expressed as normalized mRNA levels to *GAPDH* mRNA levels of triplicate determinations with pooled samples from 5 chickens. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant, NE = necrotic enteritis.

Various innate immune factors contribute to genetically predisposed NE susceptibility, although the role of antimicrobial peptides in this infection has not been investigated so far. This is the first report showing the expression of *AvBD* transcripts in the crop, intestine, and spleen from 2 commercial broiler chicken lines that show different NE susceptibility. The results showed that β -defensins were highly expressed following *E. maxima* and *C. perfringens* infections with preferential

expression of *AvBD1*, 7, 9 in the crop, *AvBD8*, 10, 13 in the intestine, and *AvBD1*, 7 in the spleen (Figures 1, 2, and 3). Interestingly, in the spleen of both commercial broiler lines that were uninfected, the levels of *AvBD1*, 2, 4, 5, 6, and 7 transcripts were significantly expressed. A constitutive expression of some β -defensins can be found in nearly all gastrointestinal tissues. Cathelicidin and certain β -defensins were induced following infection with *Helicobacter pylori*, *Campylobacter jejuni*, and

Salmonella (Milona et al., 2007; van Dijk et al., 2007; Wehkamp et al., 2007; Akbari et al., 2008). In *S. enteritidis* infection, tissue-specific β -defensin expression was reported with *AvBD1*, 2, 4, 6, and 9 in the digestive tract (Milona et al., 2007; van Dijk et al., 2007; Akbari et al., 2008; Derache et al., 2009), and *AvBD1*, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 14 in the ovary, oviduct, or sperm (Subedi et al., 2007; Das et al., 2011; Michailidis et al., 2012). Parasitic infections induced an increase in the number of enteric α -defensin-expressing Paneth and intermediate cells in the small intestine as well as some members of the β -defensin family in the distinct parts of the gastrointestinal tract in mice (Cunliffe and Mahida, 2004).

The results of this study showed that the expression patterns of *AvBD* in NE differ from those seen in *S. enteritidis* infection in commercial broiler chickens, suggesting that *AvBD8*, 10, 11, and 13 may play an important role in host intestinal defense against the pathogens of NE.

Following infection with *Salmonella*, *AvBD1* and *AvBD2* expression levels did not change even though their baseline levels were significantly higher in the *S. enteritidis*-resistant inbred chickens (Derache et al., 2009). In our results, *AvBD1* and 7 transcript levels in the crop were significantly increased in uninfected and NE-infected chickens of the Cobb chicken line that was studied. In general, Ross chickens showed higher β -defensin transcripts in the crop, intestine, and spleen compared with Cobb.

In vivo studies have shown important roles of defensins in host innate immunity to pathogens. Enhanced expression of defensin genes is usually associated with heightened inflammatory response following the infection with pathogens (Menendez and Finlay, 2007). Transient induction of proinflammatory cytokines by chicken gut microflora resulted in an activation and normalization of the innate immune system in the gut and increased resistance to *S. enteritidis* infection (Crhanova et al., 2011) and the production of antibody response was associated with Gal3 (*AvBD3*) and Gal7 (*AvBD4*) SNPs in broiler sires following *S. enterica* serovar Enteritidis vaccination (Hasenstein et al., 2006).

In humans, some β -defensins, such as *hBD1* or *hBD2*, were induced or upregulated by proinflammatory cytokines, such as IL-1 β , IL-6, and TNF- α (Scott and Hancock, 2000; McDermott, 2006). Interleukin-17, which has been shown to be important for host defense against bacterial and fungal pathogens, was induced following infection with *Salmonella* Typhimurium infection and could be involved in local induction of various defensins (Raffatellu et al., 2009).

In chickens, van Dijk et al. (2007) reported that Gal-6 (*AvBD9*) expression might be upregulated in the digestive tract via NF- κ B and activator protein 1 (AP-1) pathways. Because our previous studies showed that proinflammatory cytokines, such as IL-1 β , IL-6, IL-17F, and TNFSF15, were highly induced in the intestine of *E. maxima*-infected chickens compared with

uninfected controls following primary infection, these cytokines may be involved in the local regulation of various β -defensins, as shown in this study (Hong et al., 2006b; Park et al., 2007).

Taken together, tissue- and strain-dependent expression of *AvBD* transcripts are induced in broiler chickens following NE infection, suggesting that the local expression of these antimicrobial peptides could be important in the regulation of inflammation and host innate immunity to this infection. Further studies will be necessary to better understand the nature of host-pathogen interaction that is involved in NE infection and the role of these various *AvBD* that are induced during NE.

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