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# Expression of Antimicrobial Peptides in Cecal Tonsils of Chickens Treated with Probiotics and Infected with *Salmonella enterica* Serovar Typhimurium[▽]

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**Several strategies currently exist for control of *Salmonella enterica* serovar Typhimurium colonization in the chicken intestine, among which the use of probiotics is of note. Little is known about the underlying mechanisms of probiotic-mediated reduction of *Salmonella* colonization. In this study, we asked whether the effect of probiotics is mediated by antimicrobial peptides, including avian beta-defensins (also called gallinacins) and cathelicidins. Four treatment groups were included in this study: a negative-control group, a probiotic-treated group, a *Salmonella*-infected group, and a probiotic-treated and *Salmonella*-infected group. On days 1, 3, and 5 postinfection (p.i.), the cecal tonsils were removed, and RNA was extracted and used for measurement of avian beta-defensin 1 (AvBD1), AvBD2, AvBD4, AvBD6, and cathelicidin gene expression by real-time PCR. The expressions of all avian beta-defensins and cathelicidin were detectable in all groups, irrespective of treatment and time point. Probiotic treatment and *Salmonella* infection did not affect the expression of any of the investigated genes on day 1 p.i. Furthermore, probiotic treatment had no significant effect on the expression of the genes at either 3 or 5 days p.i. However, the expression levels of all five genes were significantly increased ($P < 0.05$) in response to *Salmonella* infection at 3 and 5 days p.i. However, administration of probiotics eliminated the effect of *Salmonella* infection on the expression of antimicrobial genes. These findings indicate that the expression of antimicrobial peptides may be repressed by probiotics in combination with *Salmonella* infection or, alternatively, point to the possibility that, due to a reduction in *Salmonella* load in the intestine, these genes may not be induced.**

Chickens may harbor food-borne pathogens, including *Salmonella enterica* serovar Typhimurium, in their intestines, and administration of live bacterial cultures in the form of probiotics can reduce intestinal colonization with these bacteria (9, 18). Probiotics have also been shown to exert other functions, such as maintenance of health and promotion of growth, in chickens (11).

In spite of considerable published data regarding the efficacy of probiotics in reducing intestinal colonization by enteric pathogens, the mechanisms of action of probiotics are not fully understood. Several mechanisms have been proposed for probiotic functions, among which modulation of the immune system has recently received attention. Probiotic bacteria can exert immunomodulatory activities through their interactions with the host immune system. These interactions may lead to enhancement of natural and antigen-specific antibodies (6, 7), activation or suppression of T cells (3, 19), and changes in cytokine expression profiles (5, 12, 29). Moreover, probiotics are able to induce the expression of antimicrobial peptides by host cells (23, 30). Collectively, the above-mentioned mechanisms contribute to the immunomodulatory activities of probiotics.

Antimicrobial peptides are a part of innate defense mechanisms (25) and are divided into two main families, defensins and cathelicidins (4). According to the positions of their disulfide bonds, defensins are subdivided into two major classes: alpha-defensins and beta-defensins (16). In addition, a class of defensins known as teta-defensins, with a circular structure and a different disulfide motif, has been identified in rhesus monkeys but not in any other species (4, 26). Defensins have a wide range of antimicrobial activity against different groups of microorganisms, including gram-negative and gram-positive bacteria, as well as fungi and certain enveloped viruses (4). In addition to their direct antimicrobial activities, defensins might also participate in regulation of acquired immune response against pathogens by facilitating maturation of dendritic cells and recruitment of effector T cells to infected tissues (34).

Avian species express only beta-defensins (15), and avian heterophils or epithelial cells have been reported to express these molecules (25). Members of the beta-defensin family in chickens are also called gallinacins. Recently, a new nomenclature was proposed for avian beta-defensins, which will be used hereafter. To date, 14 avian beta-defensins (avian beta-defensin 1 [AvBD1] to AvBD14) have been discovered (15). Among these molecules, AvBD1 and AvBD2 are expressed in different sections of the chicken intestine, lungs, and bone marrow (1, 36). AvBD6 is expressed in different parts of the digestive system, especially in the proximal part of the intestine

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(27). AvBD4 was shown to have an expression pattern similar to those of AvBD1 and AvBD2 (14).

Beta-defensin genes are inducible, and inflammation or bacterial infection in host tissues can induce the expression of these genes (16, 31). Induction of beta-defensins following infection with bacteria or their components has also been shown to occur in chickens. Yoshimura et al. (35) reported that inoculation of cell cultures of the hen oviduct with *Salmonella enterica* serovar Enteritidis and lipopolysaccharide (LPS) increased the expression levels of AvBD1, AvBD2, and AvBD3. Upregulation of AvBD1, AvBD7, and AvBD12 in the theca layer of ovarian follicles in LPS-injected laying hens has also been demonstrated (24). AvBD4 was expressed significantly in the liver of chickens in response to *Salmonella* serovar Enteritidis and serovar Typhimurium (17).

Antimicrobial properties of avian beta-defensins in chickens have been demonstrated in several studies. Higgs et al. (10) reported that AvBD13 can kill *Salmonella* serovar Typhimurium and *Listeria monocytogenes* at a concentration of 500 μg/ml. By use of an in vitro assay, it was demonstrated that AvBD6, AvBD4, and AvBD5 possess bactericidal activity against *Salmonella* species (17). In another study, the expression of AvBD6 in the cecal tonsils of 6-week-old broiler chickens and its antimicrobial activity against enteric pathogens were shown (27). Finally, antimicrobial activity of chicken cathelicidins has also been reported (2, 32). Also of interest are the observations that probiotic bacterial strains, including some strains of lactobacilli as well as a cell wall preparation of *Enterococcus faecalis* (EC-12), can induce the expression of defensins, such as AvBD2, in various tissues, including the tongue and the bursa of Fabricius (22, 23).

In our previous studies, we showed the immunomodulatory effects of a probiotic product in newly hatched chicks (5, 6, 7). Particularly, in a recent study, we examined cytokine gene expression in the cecal tonsils of chickens treated with probiotics and infected with *Salmonella* (5). The aim of the present study was to extend our previous studies and to investigate the effects of this probiotic in combination with *Salmonella* infection on innate host defenses by measuring antimicrobial peptide gene expression in the cecal tonsils of broiler chicks during their first week of life.

## MATERIALS AND METHODS

**Chicks and experimental design.** Seventy-two 1-day-old female broiler chicks were purchased from a local hatchery (Maple Leaf Food Inc., New Hamburg, ON, Canada). After arrival, the chicks were divided into four groups and randomly assigned to four separate rooms in an isolation unit (Ontario Veterinary College, University of Guelph, ON, Canada). All groups were provided with a broiler starter diet during the experimental period. All chicks had free access to feed and water during the experiment. The animal experimentations were approved by the Animal Care Committee, University of Guelph. The treatment groups were as follows: (i) the negative control (no probiotic treatment and no *Salmonella* infection), (ii) the probiotic-treated group, (iii) the *Salmonella*-infected group, and (iv) the probiotic-treated and *Salmonella*-infected group.

**Probiotic treatment and *Salmonella* infection.** The probiotic used in this study was a commercial preparation consisting of three species of beneficial bacteria: *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Enterococcus faecalis* (Intervet, Whitby, ON, Canada). Immediately after arrival, the chicks and their boxes were sampled for the presence of *Salmonella*. On the first day of age, all chicks in groups ii and iv (the probiotic-treated group and the probiotic-treated and *Salmonella*-infected group, respectively) received 0.5 ml phosphate-buffered saline (PBS) containing $1 \times 10^6$ CFU of the probiotic via oral gavage. On the following day (the second day of age), the chicks in groups iii and iv (the *Salmonella*-infected group and the probiotic-treated and *Salmonella*-infected group, respectively) were orally infected with $1 \times 10^4$ CFU of nalidixic acid-resistant *Salmonella enterica* serovar Typhimurium of phage type 193 in 0.5 ml PBS. PBS was used as a placebo in untreated and/or uninfected groups on either the first or the second day of age.

**Tissue collection and storage.** On days 1, 3, and 5 postinfection (p.i.) (3, 5, and 7 days of age, respectively), six chicks from each group were randomly selected and euthanized by cervical dislocation. The cecal tonsils were removed and kept in RNAlater (Qiagen Inc., Mississauga, ON, Canada) at −20°C until processing for RNA extraction.

**Total RNA extraction and reverse transcription.** Total RNA from cecal tonsil samples was extracted using Trizol reagent (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using 2 μg of extracted total RNA, oligo(dT)$_{12\text{-}18}$ primers, and a reverse transcription kit (Superscript first-strand synthesis system; Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The obtained cDNA was then stored at −20°C until use.

**Construction of standard DNA plasmids.** The sequences of the AvBD1, AvBD2, AvBD4, and AvBD6 beta-defensin genes were obtained from GenBank. These genes were selected because their expression in cecal tonsils or in the chicken intestine had previously been shown (1, 14, 27, 36). We also attempted to amplify AvBD3 and AvBD13 but were not successful; therefore, we did not pursue study of the expression of these genes further. In addition to beta-defensins, we also examined cathelicidin gene expression. The sequence used for amplification of the cathelicidin gene has 100% identity with that for fowlicidin1 (a chicken cathelicidin), identified by Xiao et al. (32). In order to construct standard plasmid DNA required for relative quantification of target and reference (β-actin) genes, cDNA was amplified by PCR using primer pairs specific to each gene (Table 1). Amplification of target fragment was done with a reaction mixture (25-μl total volume) containing 1 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.4 μM of each gene-specific primer, and 1 unit *Taq* DNA polymerase. The cycling parameters were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplification was terminated by a final extension at 72°C for 10 min. PCR products were then purified using a commercial kit (PCR purification kit; Qiagen Inc., Mississauga, ON, Canada) and subsequently cloned into the pDrive cloning vector (Qiagen PCR cloning kit; Qiagen Inc., Mississauga, ON, Canada). Clones were screened by restriction enzyme digestion, and positive clones were sequenced. Plasmid concentration was determined by measurement of absorbance at 260 nm. To convert the plasmid concentrations to copy numbers, the following equation was used: number of copies per μl of target plasmid DNA = DNA concentration in grams per μl $\times 6 \times 10^{23}$ copies per mol/molecular weight of the plasmid DNA in grams per mol. In order to generate standard curves, 10-fold serial dilutions ($10^{-1}$ to $10^{-9}$) of standard plasmids were made and amplified in duplicate by real-time PCR.

**Real-time PCR.** Quantifications of target and reference genes in cDNA samples were carried out by fluorometric real-time PCR using a LightCycler 480 instrument (Roche Diagnostic GmbH, Manheim, Germany) and a real-time PCR kit (LightCycler 480 DNA Sybr green I master; Roche Diagnostic GmbH, Manheim, Germany). The assay was performed using a microplate (LightCycler 480 multiwell plate 384; Roche Diagnostic GmbH, Manheim, Germany) with final volume of 20 μl/well, consisting of 0.25 μM of each gene-specific primer (Table 1), 10 μl of LightCycler 480 DNA Sybr green I master mix (containing fast-start *Taq* DNA polymerase, Sybr green I dye, and $MgCl_2$), 5 μl of the cDNA template, and PCR-grade water.

The thermal cycling protocol consisted of an initial denaturation at 95°C for 10 min, followed by amplification for 40 cycles at 95°C for 1 s, 58°C for 5 s, and 72°C for 9 to 11 s. The specificity of amplification for each product was determined by melting curve analysis at 95°C for 1 s and 65°C for 15 s, followed by progressive rising of the temperature to 95°C, with continued measurement of fluorescence, and finally cooling of the plate at 40°C for 30 s. Alongside each real-time PCR assay, a $10^{-4}$ dilution of a related standard DNA plasmid and a blank control were run to serve as a calibrator and a negative control, respectively.

**Data analysis.** The efficiency of the real-time PCR assays was calculated by the LightCycler 480 software program. Relative expression was calculated as a ratio between expression of target genes (the AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin genes) and expression of the β-actin gene (as a reference gene) in the same cDNA sample. To achieve this, the following equation, which considers differences in the efficiencies obtained for the target and reference genes, was used (20): ratio = $(E_T)^{C_PT(c) - C_PT(s)} \times (E_R)^{C_PR(s) - C_PR(c)}$. In this equation, $E_T$ and $E_R$ represent the efficiencies of real-time PCR from the target and reference genes, $C_PT(c)$ and $C_PT(s)$ represent the crossing points of the target gene for the

TABLE 1. Sequences of primer pairs used for amplification of target and reference genes

| Primer target | Orientation[a] | Sequence | Product size (bp) | Temp (°C)[b] |
|---|---|---|---|---|
| AvBD1 | F | GGTTCTTACTGCCTTGCTGT | 158 | 58 |
| | R | TGACTTCCTTCCTAGAGCCT | | |
| AvBD2 | F | GGACTGCCTGCCACATACAT | 239 | 58 |
| | R | TTGCAGCAGGAACGGAAC | | |
| AvBD4 | F | TACCTGCTGCTGTCTGTCCT | 158 | 58 |
| | R | AGTCCACTGCCACATGATCC | | |
| AvBD6 | F | TTGTGGCAGTTCATGGAG | 188 | 58 |
| | R | ACTTCTGGAGATCCTGTGC | | |
| Cathelicidin | F | CTGCACAACCTCAACTTCAC | 231 | 65 |
| | R | GTATCCTGCAATCACAGTCC | | |
| β-Actin | F | CAACACAGTGCTGTCTGGTGG | 205 | 64 |
| | R | ATCGTACTCCTGCTTGCTGAT | | |

[a] F, forward; R, reverse.
[b] The annealing duration for each primer was 5 seconds.

calibrator and the samples, and $C_PR(s)$ and $C_PR(c)$ represent the crossing points of the reference gene for the samples and the calibrator, respectively.

All data were analyzed according to a completely randomized design, consisting of four treatments and six replicates, using the General Linear Models procedure in SAS. Treatment means were compared by the new Duncan multiple-range test at an $\alpha$ value of 0.05 (SAS).

## RESULTS

The results of *Salmonella* infection and probiotic treatment effects on the expressions of AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin are illustrated in Fig. 1A to E. Irrespective of treatment group and time point, the expressions of all antimicrobial peptide genes investigated in this study (AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin) were detectable in the cecal tonsils of chicks.

The expressions of the AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin genes in the cecal tonsils of chicks were not affected by probiotic treatment or *Salmonella* infection on day 1 p.i. Furthermore, probiotic treatment had no significant effect on the expressions of these genes either at 3 or at 5 days p.i., compared to what was found for chickens that were not treated with probiotics or infected with *Salmonella*. However, the expression levels of AvBD1, AvBD2, AvBD4, AvBD6 and cathelicidin were significantly increased ($P < 0.05$) in response to *Salmonella* infection at 3 days p.i. (2.7-, 2.87-, 3.7-, 1.83-, and 2.72-fold increases for AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin, respectively, compared to control levels). However, this upregulation of genes due to *Salmonella* infection was not observed in chicks which had been treated with probiotics before being infected with *Salmonella* (the probiotic-treated and *Salmonella*-infected group). The elevated expression levels of the genes in response to *Salmonella* infection continued until 5 days p.i. (2.12-, 3.0-, 2.50-, 1.45-, and 1.39-fold increases for AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin, respectively, compared to negative-control levels). However, at this time point, the increased expression levels were significant ($P < 0.05$) only for AvBD2 and AvBD6. Similar to what was observed at 3 days p.i., the expressions of these genes were unaffected in the probiotic-treated and *Salmonella*-infected group at 5 days p.i.

## DISCUSSION

In this study, the possible role of antimicrobial peptides in *Salmonella* infection and probiotic-mediated protection against *Salmonella* in chickens was investigated. This was evaluated by measuring the expressions of AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin in the cecal tonsils of chickens infected with *Salmonella* serovar Typhimurium or treated with probiotics prior to *Salmonella* infection. Expressions of the avian beta-defensin and cathelicidin genes were detectable in all groups during the first week of age. This observation confirmed and extended the previous observations that AvBD1 and AvBD2 are expressed in the small and large intestines of healthy chicks during the first week of age (1). In addition, we showed that while *Salmonella* infection led to elevated expression of all the antimicrobial genes studied here, probiotic treatment prior to infection dampened the expression of these genes.

We have previously shown that administration of the probiotic product used in the present study could reduce cecal *Salmonella* counts by 1.3 to 3 logs (5; J. R. Chambers, H. R. Haghighi, and S. Sharif, unpublished data). In the present study, increases were observed in the expression levels of AvBD1, AvBD2, AvBD4, AvBD6, and cathelicidin genes in the cecal tonsils of the chicks in response to *Salmonella* infection on day 3 p.i. and, in the cases of some genes, on day 5 p.i. as well. However, the probiotic bacteria used in this study did not evoke any significant increase or decrease in the expression of any of the defensin genes. The increases in the expression levels of these antimicrobial genes in response to *Salmonella* infection may be an indication that all of these genes are involved in host responses to infection. In agreement with our results, Sadeyen et al. (21) also reported increases in the expression levels of AvBD1 and AvBD2 in the cecal tonsils of laying hens in response to infection with *Salmonella* serovar

*Enteritidis*. Furthermore, upregulation of AvBD1, AvBD7, and AvBD12 in the theca layer of ovarian follicles in LPS-injected laying hens has been demonstrated (24).

Probiotics may also induce the expression of beta-defensins. Wehkamp et al. (30) have reported the induction of human beta-defensin 2 in Caco-2 intestinal epithelial cells through a number of probiotic bacterial strains, such as *Escherichia coli* Nissle 1917 and some lactobacilli strains. The upregulation of the human beta-defensin 2 gene in Caco-2 cells by several probiotic *Lactobacillus* strains also has been demonstrated by Schlee et al. (23). However, there is no report of effects of live probiotic bacteria on the expression of avian beta-defensin genes in chickens. Nevertheless, it has been shown that oral administration of a cell wall preparation of *Enterococcus faecalis* strain EC-12 in newly hatched broiler chicks increased expression of Gal-2 in the tongue and the bursa of Fabricius (22).

Our results indicate that although *Salmonella* infection is able to induce the expression of avian beta-defensin and cathelicidin genes in the cecal tonsils of chickens, early intestinal colonization by probiotic bacteria does not result in enhancements in the expression levels of these genes. Moreover, when *Salmonella* inoculation was done after probiotic treatment (in the probiotic-treated and S*almonella*-infected group), the expression levels of these genes were similar to those observed in uninfected chickens. Therefore, it may be concluded that when probiotic bacteria are combined with *Salmonella*, they may have a direct antagonistic effects against *Salmonella*-induced expression of defensin genes. Alternatively, the downregulation of these genes in the probiotic-treated and *Salmonella*-infected group may be the result of inhibitory effects of probiotic bacteria on *Salmonella* colonization. Consequently, a reduction in intestinal colonization by *Salmonella* may have eliminated the inflammatory conditions needed for the induction of these genes. We have also previously observed that the same probiotic used in the present study was able to reduce intestinal colonization by *Salmonella* serovar Typhimurium as well as expression of proinflammatory cytokines, such as interleukin 12 and gamma interferon, in cecal tonsils.

Despite some reports that the expression of beta-defensins is enhanced by mixed cultures or individual probiotic bacteria (23, 30), we did not observe a significant change in the expressions of avian beta-defensins and cathelicidin genes in the group that was treated with probiotics only. Enhanced expression of defensin genes is usually associated with pathogenic infections and inflammations caused by these infections (16). Hence, probiotic bacteria, which are usually regarded as members of the commensal microbiota, may be well adapted to their host, and as a result, their colonization will not result in

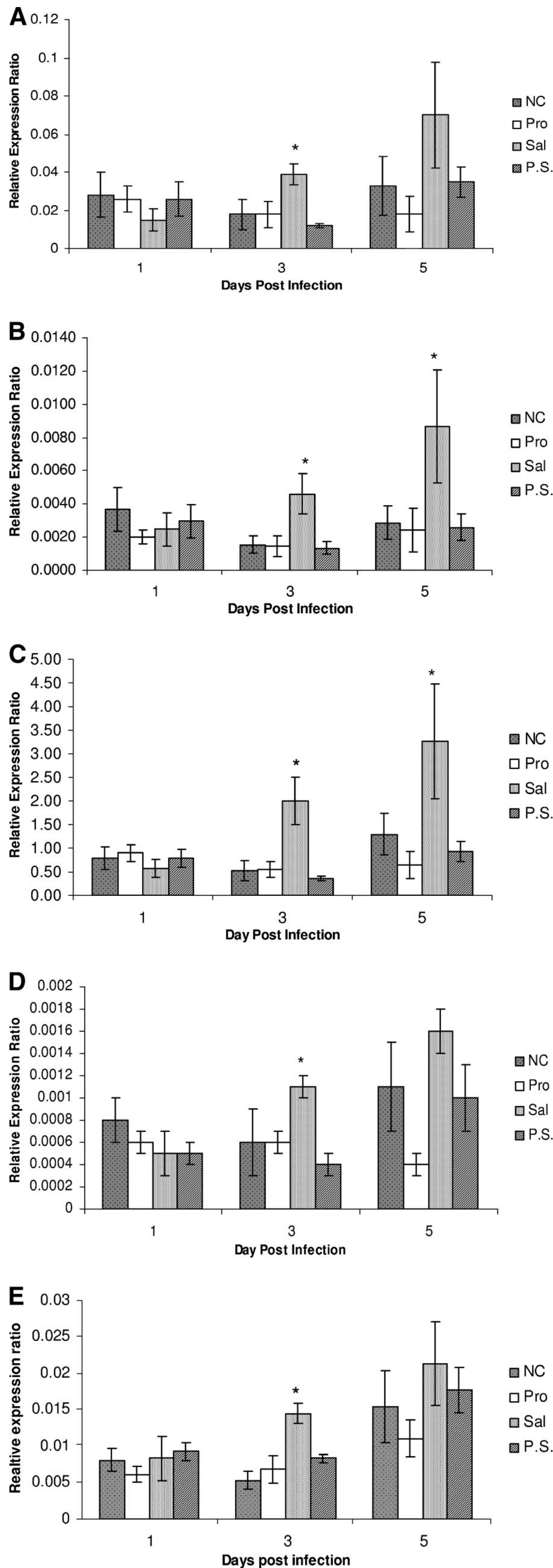


FIG. 1. Relative expression levels of AvBD1 (A), AvBD2 (B), AvBD6 (C), AvBD4 (D), and cathelicidin (E) in the cecal tonsils of chicks. The treatment groups were as follows: the negative control (NC; no probiotic treatment, no *Salmonella* infection), the probiotic-treated group (Pro), the *Salmonella*-infected group (Sal), and the probiotic-treated and *Salmonella*-infected group (P.S.). Target gene expression is presented relative to β-actin expression and normalized to a calibrator. Error bars represent standard errors of the means. The differences in gene expression among groups at each time point were tested by Duncan's new multiple range test and were considered significant at $P$ values of $\leq 0.05$ (*).

inflammation or ensuing enhancement in expression of antimicrobial peptides.

In conclusion, we have demonstrated that infection of young chicks with *Salmonella* serovar Typhimurium significantly increases the expression of several of the antimicrobial peptide genes in cecal tonsils. Furthermore, when chickens were treated with probiotics prior to *Salmonella* infection, the expressions of avian beta-defensin and cathelicidin genes were reduced to levels comparable to those seen in the negative-control group. Further studies are needed to reveal the source of antimicrobial peptides in the cecal tonsils of *Salmonella*-infected chicks as well as the mechanisms of action of probiotics in downregulating antimicrobial peptide genes in infected chickens.

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