

IMMUNOLOGY, HEALTH AND DISEASE

Effect of antibiotic treatment on microbial composition and expression of antimicrobial peptides and cytokines in the chick cecum

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ABSTRACT The aim of this study was to confirm whether the expression of innate immune molecules in the chick cecum is altered in association with changes in the composition of the intestinal microbiome that are regulated by treatment with antibiotics. Broiler chicks were administered with antibiotics (penicillin and streptomycin) daily, and the composition of the microbiota, expression of innate immune molecules, and localization of antimicrobial peptides in the chick cecum were examined at day 7 and day 14 using real-time PCR and immunohistochemistry. The oral administration of antibiotics caused an increase in the relative frequency of the Enterobacteriaceae family and a decrease in some gram-negative (Barnesiellaceae) and gram-positive bacterial (Clostridiaceae and Erysipelotrichaceae) families. The gene expression levels of immune molecules, including 4 Toll-like receptors (**TLR**) (TLR 2, 4, 5, and

21), inflammation-related cytokines (IL-1 β , TGF β 3, TGF β 4, and IL-8), and antimicrobial peptides (avian β -defensins and cathelicidins) showed a tendency to decrease with antibiotic treatment at day 7. However, expression levels of TLR21 and some cytokines (IL-1 β , TGF β 3, and IL-8) were higher in the cecum or cecal tonsils of the antibiotic-treated group than in those of the control at day 14. The immunoreactive avian β -defensin 2 and cathelicidin 1 proteins were localized in the leukocyte-like cells in the lamina propria, and they were aggregated in the form of small islands. We conclude that the expression of innate immune molecules, including TLR, inflammation-related cytokines, and antimicrobial peptides, in the cecum are altered in association with changes in the density or composition of the luminal microbiota during the early phase of life in chicks.

Key words: broiler chick, intestinal immune system, innate immune molecule, antibiotic, intestinal microbiota complex

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INTRODUCTION

The gut contains numerous microorganisms including beneficial and commensal microbiota, and the relationship between these microbiota and the immune system allows the host to maintain the balance between active immunity to pathogens and tolerance to self-antigens and food antigens (Vlasova et al., 2019). Occasionally, some pathogenic agents such as *Salmonella* and *Campylobacter* bacteria, which may cause vertical infection in chicks and human food-borne diseases, may

appear in the intestinal microbiota (Kaufman et al., 2008). Thus, enhancing immunodefense functions in the chick intestine is required for the production of healthy chicks and safe products. Acquired immunity develops with the maturation of the lymphoid system during the first few weeks of life (Bar-shira et al., 2003). Immunodefense by innate immunity plays a crucial role in preventing infection in the chick intestinal mucosa before the maturation of the lymphoid system.

In the process of innate immune response, Toll-like receptors (**TLR**) recognize microbe-associated molecular patterns, leading to the synthesis of proinflammatory cytokines and antimicrobial peptides (**AMP**). The expression of TLR2, TLR4, TLR5, and TLR21 that recognize bacterial microbe-associated molecular patterns has been identified in the gut mucosa of chicks (MacKinnon et al., 2009; Rajput et al., 2017). Not only proinflammatory cytokines but also AMPs including

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avian β -defensins (**AvBD**) and cathelicidins (**CATH**) are expressed in the chick ileum and cecum (Mohammed et al. 2016; Terada et al., 2020b). We revealed that exposure of the intestinal mucosa to TLR ligands, including Pam3CSK4, lipopolysaccharide, and CpG-oligodeoxynucleotides, affected the expression of proinflammatory cytokines and AvBD (Terada et al., 2020a). Taha-Abdelaziz et al. (2017) reported that the gene expression levels of proinflammatory cytokines, chemokine, and AMP in the chick intestine were increased, whereas those of anti-inflammatory cytokines (*IL-10* and *TGF β 4*) were decreased with the oral administration of CpG-oligodeoxynucleotides and lysis of heat-killed *Campylobacter jejuni*. Crhanova et al. (2011) reported that the complexity of gut microbiota gradually increased from day 1 to day 19 of life and showed a higher expression of AvBD for the first 3 D of life that decreased thereafter with a transient increase in *IL-8* and *IL-17* expression on day 4 of life in the chick cecum. Wisselink et al. (2017) reported that administration of antibiotics (**AB**) later in the life of chicks (day 15 to 20 post-hatch) temporarily affected the composition and diversity of intestinal microbiota and immune parameters including that of *IL-1 β* , *IL-8*, and some other cytokines. In mammals, it is described that luminal and mucosa-associated commensal bacteria modulate the host immune homeostasis, and in turn, the signals from immune cells affect the composition of microbiota (Sansone et al., 2004; Ivanov and Littman, 2011). Brown et al. (2016) reported that the ingestion of feed mixed with AB modulated the composition of intestinal microorganisms and the expression of cytokines and AMP in the mice colon. These reports suggest that the chick intestine expresses innate immune molecules including AMP and proinflammatory and anti-inflammatory cytokines, and luminal microbiota may affect the expression of these innate immune molecules in the immune system in different animals.

Previous studies have demonstrated changes in the complexity of gut microbiota and expression of immune factors in chicks during their natural growth and in those treated with AB later in life (Pedroso et al. 2006; Wise et al., 2007; Crhanova et al., 2011). However, the effect of the microbiome on the intestinal immune system in young chicks remains to be investigated further. It is necessary to understand the mechanism by which the expression of innate immune factors is regulated in the chick intestine. Thus, the goal of this study was to confirm whether the expression of innate immune molecules, including that of TLR, AMP, and proinflammatory and anti-inflammatory cytokines, in the chick cecum are altered in association with changes in the composition of the intestinal microbiome that are regulated by treatment with AB.

MATERIALS AND METHODS

Experimental Birds and AB Treatment

Broiler chicks (Chunky) were obtained by incubating fertilized eggs purchased from a local hatchery (Fukuda

Breeder, Okayama, Japan), and male chicks were selected by feather sexing. They were maintained in a brooding room under conditions of 23 h light and 1 h dark for 7 D, followed by 20 h light and 4 h dark for the next 7 D. They were fed a commercial starter diet (Nichiwa Sangyo Co. Ltd., Hyogo, Japan) and water ad libitum. The newly hatched chicks were orally administered with PBS or AB (control and AB groups, respectively). Antibiotic solution was prepared by dissolving 0.66 g streptomycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 1,000 U penicillin (Meiji Seika Pharma Co., Ltd.) in 1 mL PBS. Some chicks were administered 500 μ L AB solution or PBS daily from day 1 to 6 (sample collection was at day 7), and other chicks were administered 500 μ L solution from day 1 to 7 followed by 1 mL solutions from day 8 to 13 (sample collection was at day 14). At day 7 and 14, chicks were euthanized using carbon dioxide to collect the cecum tissues and its luminal content ($n = 10$ in the control and AB groups at day 7 and 14). The cecum and cecal tonsils tissues were used for RNA extraction and histological analysis, and the cecum luminal content was used for bacterial DNA extraction. This study was approved by the Animal Research Committee of Hiroshima University (No. C15-16).

Real-Time PCR Analysis for Immune Molecules

Total RNA was extracted from the cecum and cecal tonsils using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan), and reverse transcription to prepare cDNA was performed using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) in accordance with the manufacturer's instructions. The prepared cDNA samples were stored at -20°C until use. Real-time PCR was performed using the AriaMx Real-Time PCR System (Agilent Technologies Japan, Ltd., Tokyo, Japan). The reaction mixture (10 μ L) comprised 1 μ L cDNA, 1 \times Brilliant III SYBR Green QPCR Mix (Agilent Technologies Japan, Ltd.), 0.25 μ mol of each primer, and water. The sequences of primers used in this study are shown in Supplementary Table 1. Toll-like receptors 2-1, 4, 5, and 21 were selected as the receptors which recognize bacterial molecular patterns. *IL-1 β* , *TGF β 3* and 4, and *IL-8* were selected as the major cytokines that regulate the inflammatory response. The expression of *AvBD1*, 2, 4, 6, 7 and *CATH1/3*, 2, and 3 whose PCR products showed high densities was examined for the expression of AMP. Two thermal protocols (2 or 3 steps) were used for PCR, depending on the target gene. The protocol of 2 steps was 50 cycles at 95°C for 5 s and 58°C (*TGF β 4*), 60°C (*RPS17*, *TGF β 3*, *CATH1/3*, 2, and 3, *TLR2-1*, 4, 5, and 21), or 62°C (*AvBD2*, 4, 6, and 7) for 10 s each. The protocol of 3 steps was 50 cycles at 95°C for 5 s and 55°C (*AvBD1*) and 72°C for 10 s. Real-time PCR data were analyzed using the $2^{-\Delta\Delta\text{ct}}$ method to calculate the relative levels of gene expression in each sample and were expressed as ratios in relation to

the RPS17 housekeeping gene (Livak and Schmittgen, 2001). An RNA sample of a PBS-treated (control) chick at day 7 and day 14 was used as the standard sample.

Immunohistochemistry

Antiserum to AvBD2 and CATH1 was raised by immunization of rabbits with keyhole limpet hemocyanin-conjugated synthetic peptide (Medical & Biological Laboratories, Nagoya, Japan). The sequence of the synthetic AvBD2 peptide was CPSHLIKVGS and that of the synthetic CATH1 peptide was C-C6-YRAIKKK. The antibodies in the serum were purified using an affinity column (HiTrap NHS-activated HP; GE Healthcare Japan, Tokyo, Japan) conjugated with each synthetic peptide.

The cecum and cecal tonsils were fixed in 10% (v/v) formalin in PBS and processed for paraffin sections (4 μ m in thickness). After deparaffinization, they were autoclaved at 121°C for 15 min in 10 mmol Tris buffer with 0.1% Tween 20 (pH 10.0) for AvBD2 staining or in 10 mmol Tris buffer (pH 10.0) for CATH1 staining for antigen retrieval. After cooling and washing with PBS (5 min \times 3 times), the sections were incubated with 1% (w/v) blocking reagent (Roche Co., Basel, Switzerland) for 30 min to block nonspecific binding of antibody, followed by incubation with AvBD2 antibody diluted at 5 μ g/mL or CATH1 antibody diluted at 20 μ g/mL in PBS at 4°C for 16 h. The sections were then washed with PBS (5 min \times 3 times) and incubated with the biotin-conjugated anti-rabbit IgG (1:200) and avidin-biotin-peroxidase complex (1:50) for 1 h each using the VECTASTAIN ABC Kit (Vector Laboratories, Inc., Burlingame, CA). The immunoreaction products were visualized using a reaction mixture of 0.02% (w/v) 3,3'-diaminobenzidine-4HCl and 0.05% (v/v) H₂O₂. Then, these sections were counterstained with hematoxylin and covered after dehydration. Negative control staining was performed to confirm the specificity of immunostaining by replacing the primary antibodies with absorbed antibodies. The absorbed antibodies were prepared by incubating AvBD2 or CATH1 antibodies with corresponding peptides at a ratio of 1:5 by weight.

Intestinal Microbiome Analysis

The luminal content in the cecum of AB and control groups at day 7 and day 14 were collected and stored at -80°C until use (n = 4 each). The total bacterial DNA in these samples was extracted using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) in accordance with the manufacturer's instructions. The extracted samples were examined for microbiota analysis using 16SrRNA amplification through the MiSeq (Illumina, San Diego, CA) sequencing platform of Bioengineering Lab Co., Ltd. (Kanagawa, Japan). The operational taxonomic unit of each sample was used for taxonomic analysis, and the data were presented as the relative frequencies at the family level.

Statistical Analysis

The significance of differences in gene expression between the control and AB groups was examined using the unpaired *t*-test when the values showed normal distributions within each treatment group. If they showed non-normal distributions, the Mann-Whitney U test was used to examine the differences between the 2 groups. Differences were considered significant when the *P*-value was <0.05.

RESULTS

Figure 1A shows the relative frequency of microbiome families in the cecum of chicks of the control and AB groups at day 7 and day 14. Ruminococcaceae ([4], the number included with the name of the bacterial family in Figure 1), Lachnospiraceae [5], and Lactobacillaceae [7] showed a relatively high frequency in all chicks of the control and AB groups at day 7 and 14, except that the frequency of Lactobacillaceae [7] was negligible in 3 of the 4 chicks at day 7 in the AB group. In the control group, microbiota diversity increased at day 14 in comparison with that at day 7; Clostridiaceae [6] and Barnesiellaceae [8] were identified at day 14 but were negligible at day 7. Treatment with AB resulted in the increase in the frequency of Enterobacteriaceae [2] and decrease in that of Erysipelotrichaceae [3] at day 7 and 14 and a decrease in the frequency of Barnesiellaceae [8] and Clostridiaceae [6] at day 14 in comparison with that in the control group (Figure 1B).

The gene expression levels of *TLR* in the cecum and cecal tonsils of the control and AB groups are shown in Figure 2. At day 7, the expression level of 4 *TLR* (*TLR2-1*, *TLR4*, *TLR5*, and *TLR21*) in the cecum was not significantly different between the control and AB groups, whereas the expression levels of these 4 *TLR* in the cecal tonsils were significantly lower in the AB group than that in the control group (Figures 2A, 2C, 2E, 2G). At day 14, the expression level of *TLR21* in the cecum of the AB group was significantly higher than that in the control group (Figure 2H). However, the expression of other *TLR* in the cecum and of the 4 *TLR* in the cecal tonsils did not show a significant difference between the AB and control groups (Figures 2B, 2D, 2F, 2H).

Figure 3 shows the gene expression levels of proinflammatory and anti-inflammatory cytokines and of chemokine in the cecum and cecal tonsils of the control and AB groups. At day 7, the expression levels of *IL-1 β* and *IL-8* in the cecum were lower in the AB group than that in the control group (Figures 3A, 3G). The expression levels of *TGF β 3* and *TGF β 4* in the cecum (Figures 3C, 3E) and of all 4 cytokines in the cecal tonsils (Figures 3A, 3C, 3E, 3G) were not significantly different between the control and AB groups. At day 14, the expression levels of *TGF β 3* and *IL-8* in the cecum of the AB group were higher than those in the control group (Figures 3D, 3H) and those of *IL1- β* and *IL-8* in the cecal tonsils of the AB group were also higher than those in the control group (Figure 3H).

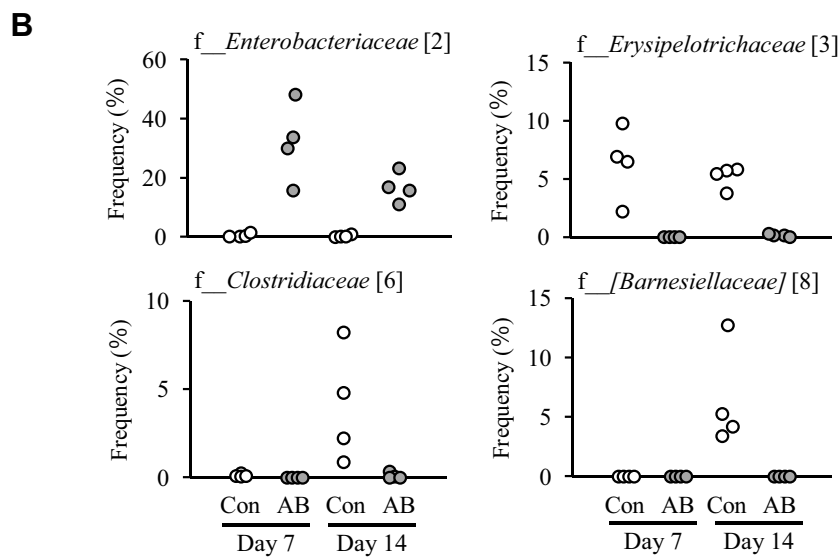
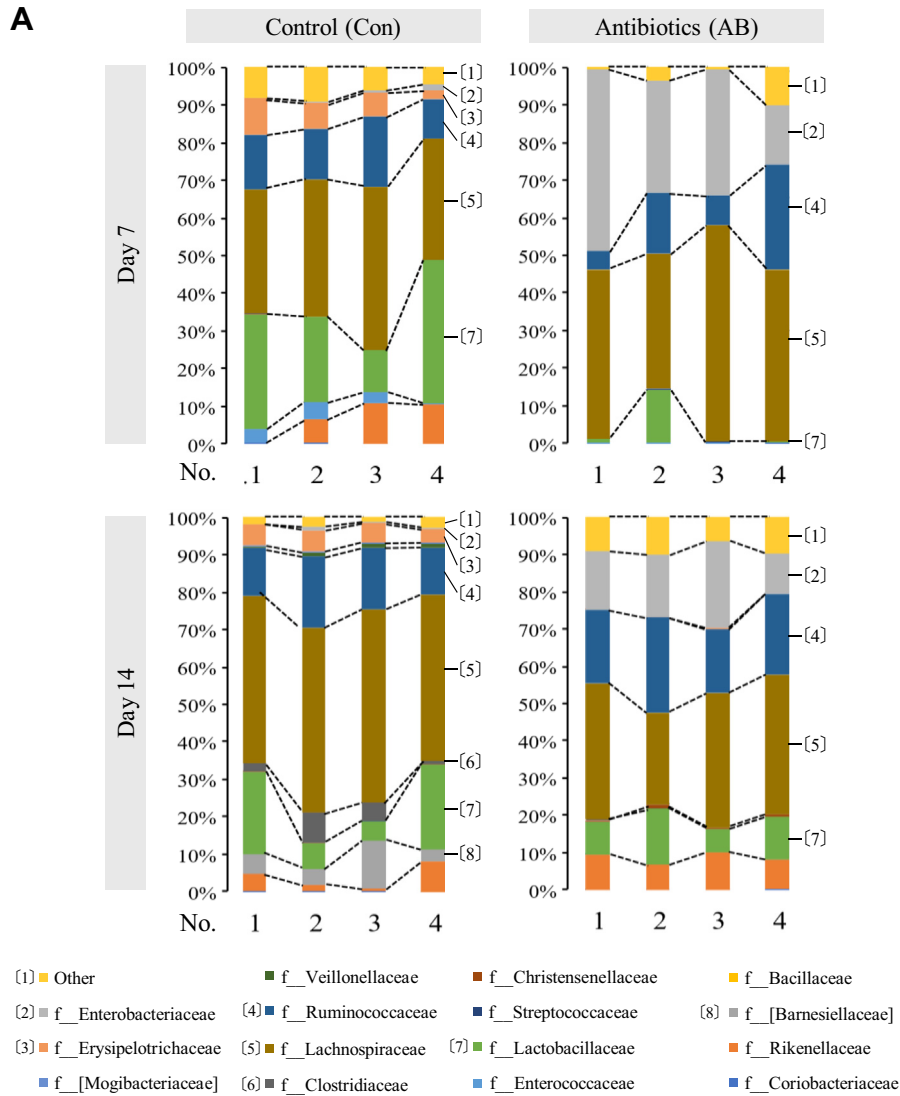


Figure 1. Microbiome profiles in the cecum contents of the chicks in the control (Con) and antibiotics (AB) groups at day 7 and day 14. (A) Relative abundance of predominant bacteria at the family level. Four cecum content samples in each group were randomly selected (No. 1–4) and analyzed for taxonomic analysis using 16SrRNA amplification. (B) Relative frequencies of 4 cecum microbiota families selected based on the appearance of marked changes with chick growth and antimicrobial treatment. The values of dots are the frequency of bacterial families in the cecum content of each chick (n = 4 for each group). Nondetection is shown as “value = 0.”

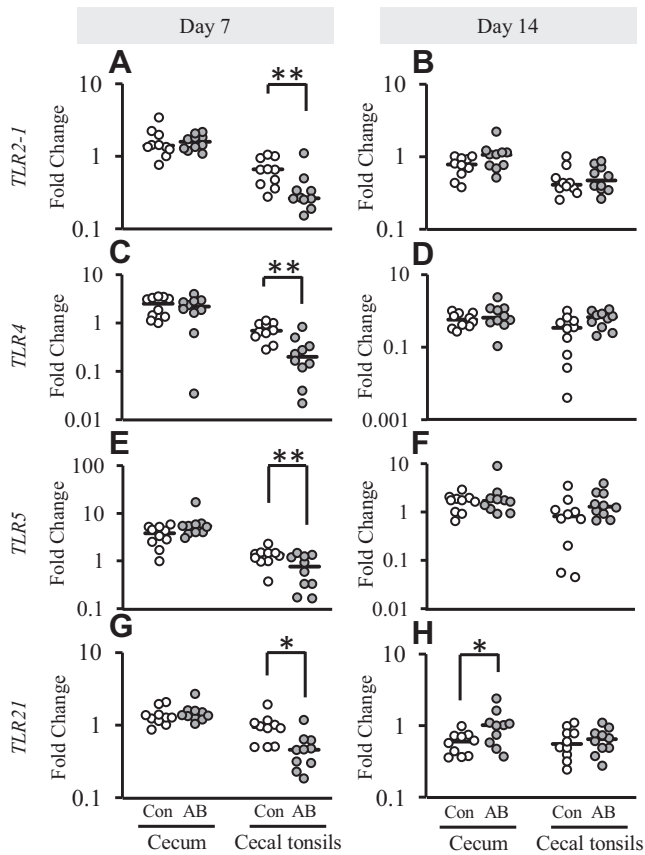


Figure 2. Effect of antibiotics administration on the expression of *TLR* in the chick cecum and cecal tonsils. Chicks were administered PBS (Con) or antibiotics (AB), and tissues were collected at day 7 or day 14 ($n = 10$ for each group). (A, C, E, G) Expressions of *TLR2-1*, *4*, *5*, and *21* at day 7; (B, D, F, H) expressions of *TLR2-1*, *4*, *5*, and *21* at day 14. The values of dots are fold changes in gene expression. The solid bar represents the median value within each group. Asterisks indicate significant differences between the Con and AB groups, determined by the Student's *t*-test or the Mann-Whitney U test ($*P < 0.05$, $**P < 0.01$). Abbreviation: TLR, Toll-like receptor.

The gene expression levels of *AvBD* in the cecum and cecal tonsils of the control and AB groups are shown in Figure 4. In the chicks at day 7, the expression levels of *AvBD1* and *AvBD2* in the cecum of the AB group were significantly lower than those in the control group (Figures 4A, 4C); however, no difference was found in the expression of other *AvBD* in the cecum between the 2 groups. Five *AvBD* in the cecum at day 14 and in the cecal tonsils at day 7 and day 14 did not show a significant difference between the AB and control groups (Figures 4A–4J).

Figure 5 shows the gene expression levels of *CATH* in the cecum and cecal tonsils of the control and AB groups. At day 7, the gene expression level of *CATH1/3* in the cecum of the AB group was significantly lower than that in the control group (Figure 5A). However, expression levels of other *CATH* in the cecum at day 7 and 3 *CATH* in the cecum and cecal tonsils at day 14 were not significantly different between the AB and control groups (Figures 5A–5J).

The localization of immunoreactive *AvBD2* and *CATH1* in the cecum and cecal tonsils of day 7 chicks

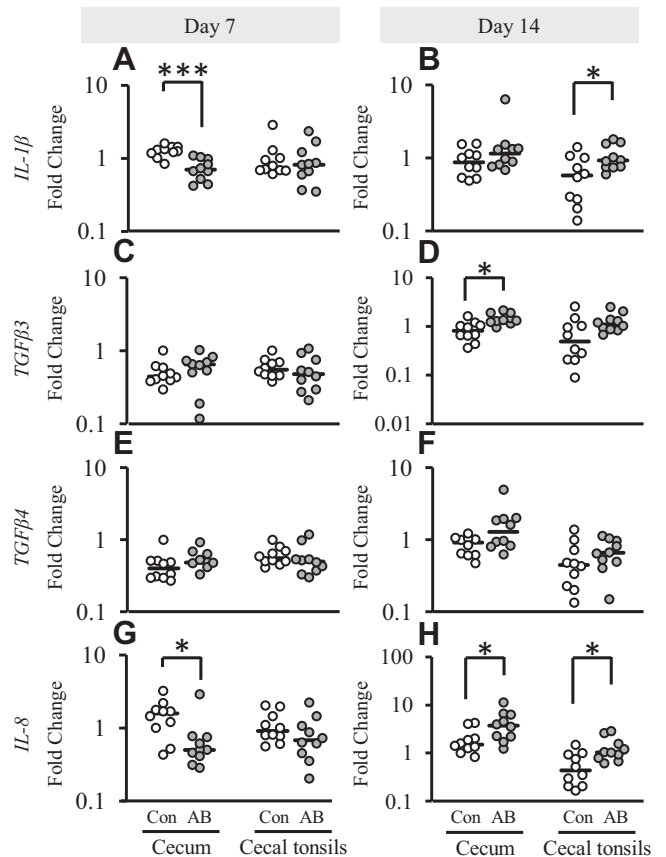


Figure 3. Effect of antibiotic administration on the expression of proinflammatory and anti-inflammatory cytokines in the chick cecum and cecal tonsils. Chicks were administered PBS (Con) or antibiotics (AB), and tissues were collected at day 7 or day 14 ($n = 10$ for each group). (A, C, E, G) Expressions of *IL-1β*, *TGFβ3*, *TGFβ4*, and *IL-8* at day 7; (B, D, F, H) expressions of *IL-1β*, *TGFβ3*, *TGFβ4*, and *IL-8* at day 14. Refer to Figure 2 for other explanations. Asterisks indicate significant differences between the Con and AB groups, determined by the Student's *t*-test or the Mann-Whitney U test ($*P < 0.05$, $***P < 0.001$).

in the control group is shown in Figure 6. The immunoreactive *AvBD2* and *CATH1* were localized in the leukocyte-like cells in the lamina propria, and they were aggregated in the form of small islands. This localization of *AvBD2*-positive cells and *CATH1*-positive cells was observed similarly in the cecum and cecal tonsils in both the control and AB groups at day 7 and 14 (data not shown).

DISCUSSION

Treatment with AB (penicillin and streptomycin) affected the expression of innate immune molecules and composition of microbiota in the chick cecum. The major findings were that 1) the oral administration of AB caused an increase in the relative frequency of the Enterobacteriaceae family and a decrease in some gram-negative (Barnesiellaceae) and gram-positive bacterial (Clostridiaceae and Erysipelotrichaceae) families; 2) the gene expression levels of immune molecules, including that of 4 *TLR*, some cytokines, and AMP, showed a tendency to decrease with AB treatment at day 7, whereas

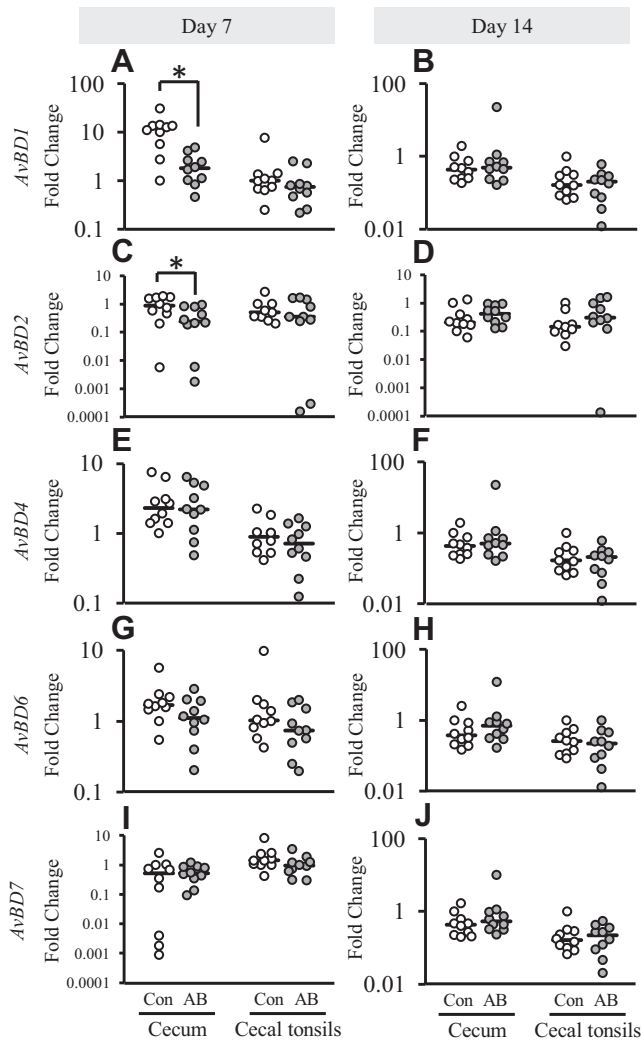


Figure 4. Effect of antibiotics administration on the expression of *AvBD* in the chick cecum and cecal tonsils. Chicks were administered PBS (Con) or antibiotics (AB) and tissues were collected at day 7 or day 14 ($n = 10$ for each group). (A, C, E, G, I) Expressions of *AvBD1*, 2, 4, 6, and 7 at day 7; (B, D, F, H, J) expressions of *AvBD1*, 2, 4, 6, and 7 at day 14. Refer to Figure 2 for other explanations. Asterisks indicate significant differences between the Con and AB groups, determined by the Student's *t*-test or the Mann-Whitney U test ($*P < 0.05$, $***P < 0.001$). Abbreviation: AvBD, avian β -defensin.

expression of *TLR21* and some cytokines was higher in the AB group than that in the control at day 14.

In this study, the composition of the microbiome in the chick cecum content at day 7 and 14 were markedly changed by the administration of AB. The major bacterial families affected by AB were the Enterobacteriaceae family showing a higher frequency in the AB group at day 7 and 14 and some gram-negative and gram-positive bacterial families (Barnesiellaceae, Clostridiaceae, and Erysipelotrichaceae) showing a decrease in frequency in the AB group. This finding is consistent with that of the study by Brown et al. (2016) who showed a significant modulation of mice colonic microorganisms with AB treatments. The expression levels of immune molecules were also affected by treatment with AB; namely, the expression levels of 4 *TLR*, some cytokines, and AMP in the AB group were lower than

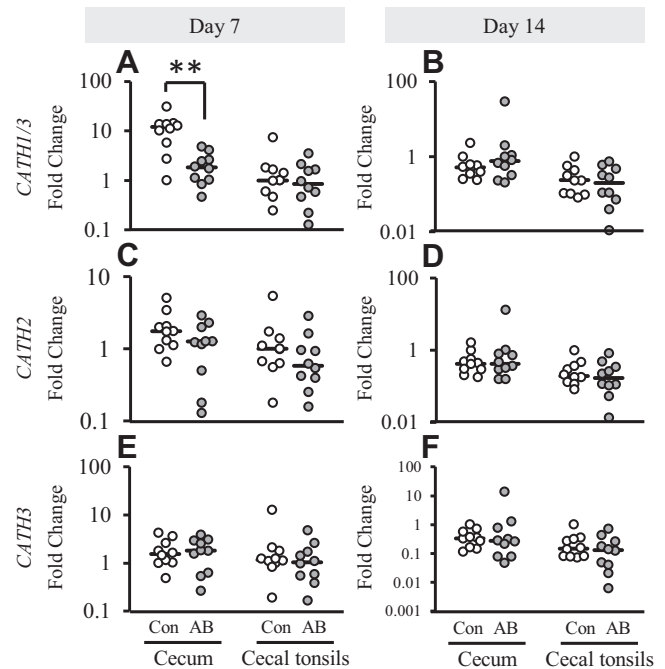


Figure 5. Effect of antibiotics administration on the expression of *CATH* in the chick cecum and cecal tonsils. Chicks were administered PBS (Con) or antibiotics (AB), and tissues were collected at day 7 or day 14 ($n = 10$ for each group). (A, C, E) Expressions of *CATH1/3*, 2, and 3 at day 7; (B, D, F) expressions of *CATH1/3*, 2, and 3 at day 14. Refer to Figure 2 for other explanations. Asterisks indicate significant differences between the Con and AB groups, determined by the Student's *t*-test or the Mann-Whitney U test ($*P < 0.05$, $***P < 0.001$). Abbreviation: CATH, cathelicidin.

those in the control group at day 7, whereas the expression of *TLR21* and some cytokines in the AB group was higher at day 14. Thus, it is likely that there are associations between the expression profiles of immune molecules and the changes caused by AB in the composition and richness of intestinal microbiota. This result partially supports Wisselink et al. (2017) who showed that administration of AB later in the life of chicks (from day 15 after hatch) affected the diversity of intestinal microbiota together with immune parameters (*IL-1 β* , *IL-8*, *IFN γ* , *IL-2*, and *IL-4*), although the effects appeared only temporarily. The specific bacteria that affect the expression of immune molecules remain to be determined. However, we suggest that the major bacterial families whose frequencies were affected by AB might participate in altering the expression of these immune molecules.

The treatment with antibiotics downregulated the expression of *TLR2-1*, 4, 5, and 21 in the cecal tonsils at day 7 and upregulated the expression of *TLR21* in the cecum at day 14 (Figure 2). Inoue et al. (2017) suggested that the expressions of *TLR2* and *TLR4* in the small intestine of mice were increased during the development of intestinal microorganisms. Thus, the different expression profiles of *TLR* between the AB and control groups may suggest that expression of *TLR* in the cecum and cecal tonsils are affected by the composition or richness of the intestinal microbiota. The decrease in the expression of *TLR2-1*, 4, 5, and 21 in the AB group

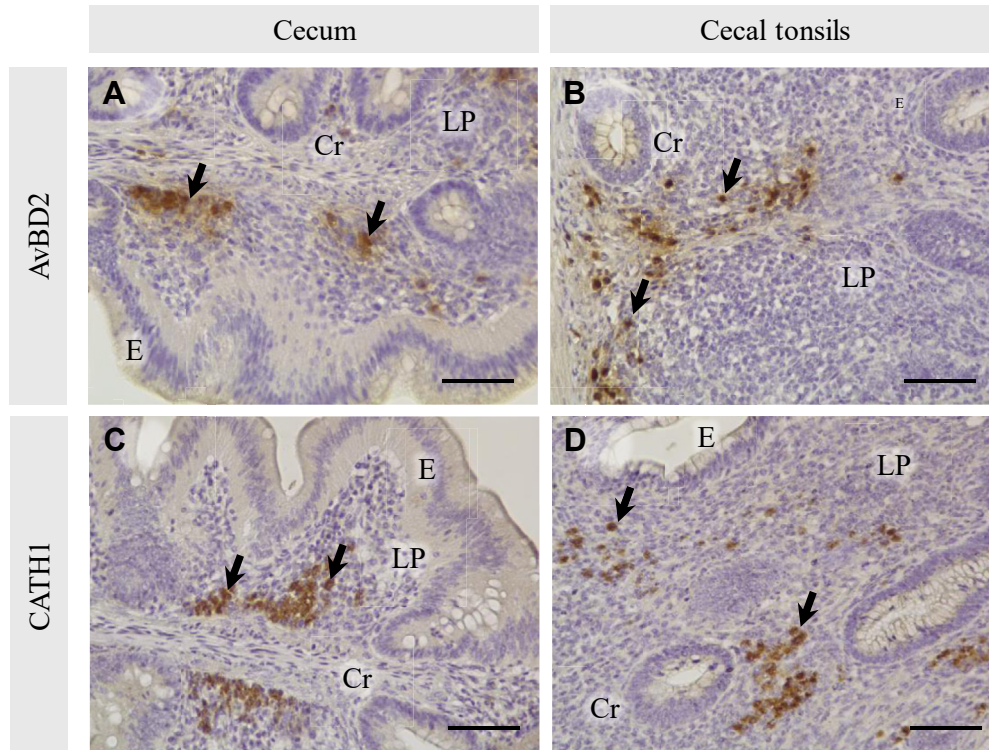


Figure 6. Micrographs showing (A, B) AvBD2 and (C, D) CATH1 immunoreactive cells in the cecum and cecal tonsils of chicks of the control group at day 7. AvBD2- and CATH1-immunoreactive cells are localized in the lamina propria in each tissue (arrows). Abbreviations: AvBD, avian β -defensin; CATH, cathelicidin; Cr, crypt; E, mucosal epithelium; LP, lamina propria. Scale bar = 50 μ m.

was identified only in the cecal tonsils at day 7, suggesting that modulation of the microbiota complex by AB reduced the expression of these TLR. However, differences in their expression between the AB and control groups were not identified in the cecum at day 7. Thus, the expression of TLR may be affected by microbiota more in the cecal tonsils than in the cecum. The expression of TLR in the cecal tonsils may be particularly important, as cytokines produced through TLR signals may play roles in the development of the lymphoid system in this tissue of healthy chicks. It is likely that the sensitivity to microbiota for TLR expression in the cecal tonsils disappeared by day 14 after hatch.

The present study showed a downregulation in the expression of proinflammatory cytokines (*IL-1 β* and *IL-8*) in the cecum by AB treatment at day 7, whereas the expression levels of proinflammatory and anti-inflammatory cytokines (*IL-1 β* , *IL-8*, and *TGF β 3*) in the cecum and cecal tonsils were upregulated by AB treatment at day 14 (Figure 3). Thus, the changes in the expression of cytokines seem not to correspond with the expression of *TLR* that was decreased in the cecal tonsils at day 7 by AB treatment. We assume that changes in the composition of microbiota caused by AB might affect the immune response system in which proinflammatory and anti-inflammatory cytokines are expressed even though the expression of *TLR* was not changed.

It was shown that AvBD2- and CATH1-positive reactions were localized in the leukocyte-like cells in the lamina propria of the cecum and cecal tonsils of chicks (Figure 6). Previous studies have shown the presence

of AvBD2 and CATH2 in the heterophil-like cells in the intestinal mucosa of chicks (Cuperus et al., 2016; Terada et al., 2018), and AvBD1, 2, and 7 proteins were identified in heterophils in the bone marrow of chickens (Derache et al., 2009). Therefore, it is probable that heterophils are 1 of the cells responsible for the expression of AvBD1, AvBD2, and CATH1. However, it is accepted that *IL-1 β* stimulates the synthesis of other inflammatory cytokines and chemokines, including *IL-8* (Dinarello et al., 2009), and *IL-8* recruits leucocytes to inflammatory tissues owing to its chemotaxis property (Rychlik et al., 2014). Our previous studies suggested that *IL-1 β* induced the expression of *AvBD* and *CATH* in the ovarian and oviduct tissues (Abdelsalam et al., 2011; Sonoda et al., 2013; Abdel-Magded et al., 2017). In the present study, the gene expression levels of *AvBD1*, *AvBD2*, and *CATH1* in the cecum were downregulated by AB treatment at day 7 (Figures 4 and 5). Thus, it is assumed that expression of these AMP decreased in association with the changes in the composition of microbiota together with the decrease in the expression of *IL-1 β* and *IL-8* in the cecum of the AB group at day 7. However, no differences were identified in the expression of *AvBD* and *CATH* between the AB and control groups in the cecal tonsils at day 7 and in the cecum and cecal tonsils at day 14. We assume that the expression of some AMP, including *AvBD1*, *AvBD2*, and *CATH1*, may be directly or indirectly modulated by the luminal microbiota in the cecum in the early phase of life in chicks, namely till day 7. However, changes in the composition of microbiota may

become less effective in altering the expression of these AMP with the growth of chicks thereafter.

In conclusion, this study showed that the expressions of proinflammatory cytokines and AMP in the cecum and cecal tonsils are altered in association with the changes in microbiota that are regulated by AB. We suggest that the expression of proinflammatory and anti-inflammatory cytokines and AMP are affected by luminal microbiota in the cecum at the early phase of life in chicks. Our findings may support the development of materials and technologies that enhance the innate immune function in the intestine of young chicks via the microbiome modification in the future studies.

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Conflict of Interest Statement: The authors declare that they have no conflict of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <http://doi.org/10.1016/j.psj.2019.11.070>.

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