

Changes in the Expression of Avian β -defensins (AvBDs) and Proinflammatory Cytokines and Localization of AvBD2 in the Intestine of Broiler Embryos and Chicks during Growth

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The aim of this study was to determine the changes in the expression of avian β -defensins (AvBDs) and proinflammatory cytokines and localization of AvBD2 in the intestine of broiler embryos and chicks during growth. The ileum and cecum of embryonic day 19 (ED19) and of day-old (D0) and 7-day-old (D7) chicks were collected. Gene expression levels of 10 AvBDs (*AvBD1*–8, 10, and 12) and proinflammatory cytokines (*IL-1 β* , -6, and -8) were analyzed using real-time PCR, and the localization of AvBD2 was examined by immunohistochemistry. Gene expression levels of *AvBD1*, 2, 6, and 7 in the ileum and of *AvBD1* and 4 in the cecum were higher on ED19 than on D7. The expression of *AvBD10* in the ileum was higher on D0 than on ED19, whereas the expression levels of *AvBD8* and 10 in the cecum were higher on D0 than on ED19, and that of *AvBD10* decreased on D7. The expression levels of *IL-1 β* , -6, and -8 in the ileum were higher on D7 than on ED19. The expression levels of *IL-1 β* , -6, and -8 in the cecum were higher on D0 than on ED19, and that of *IL-1 β* and -6 declined on D7. AvBD2-positive cells were localized in the lamina propria beneath epithelial cells of villi and crypts. The number of positive cells in the cecum mucosa was greater on D0 than on ED19 and D7. In conclusion, we suggest that AvBDs are expressed in the ileum and cecum of embryos and chicks at high levels before or just after hatching and decrease by D7. The expression of proinflammatory cytokines in the ileum increases with growth until D7, but is the highest in the cecum around hatching. These AvBDs and proinflammatory cytokines may play roles in host defense in the intestinal mucosa of embryos and neonatal chicks.

Key words: avian β -defensin, broiler embryo and chick, innate immunity, intestine, proinflammatory cytokines

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Introduction

The intestine is one of the organs that have a high risk of infection by pathogenic agents. Numerous microbes colonize the cecum soon after hatching (Crhanova *et al.*, 2011). The mucosal innate immune system plays an essential role in defense against microbial infections (Smith and Beal, 2008). The gut-associated lymphoid tissues contain functionally immature T and B cells at the time of hatching, and their functionality is attained during the first 2 weeks post hatching (Bar-Shira *et al.*, 2003). Thus, the adaptive immune system of the neonate is relatively immature, and maternal antibodies in the yolk transported to the chick are believed to play a role in protection against infection (Kovacs-Nolam

and Mine, 2012). As it is generally accepted that the innate immune system develops at an early phase, it may play a primary role in the defense against infection in the embryo and chick.

Defensins are factors involved in innate immunity that has antimicrobial activity against a variety of microbes, including Gram-negative and -positive bacteria, enveloped viruses, and fungi (Ganz, 2003). In the chicken, fourteen avian β -defensins (AvBD1 to AvBD14) have been identified (Lynn *et al.*, 2007). Lee *et al.* (2016) reported the antimicrobial activity of AvBD2-4, 6, 7, 11, and 13 against *E. coli*. Yacoub *et al.* (2015) reported that AvBD4 and 10 have antimicrobial activities against various bacteria and fungi. AvBD genes are expressed in the intestine, and are upregulated by Salmonella infection (Houng *et al.*, 2012). Proinflammatory cytokines also play essential roles in immunomodulation and inflammation (Antonopoulos *et al.*, 2017). Interleukin (IL)-1 β induces the expression of chemokines and nitric oxide, and the recruitment of immunocompetent cells in inflam-

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matory tissues (Dinarello, 2009). IL-6 is a multifunctional cytokine that regulates antibody synthesis (Chomarat *et al.*, 2000). We have reported that IL-1 β and IL-6 also upregulate the expression of AvBDs in the oviduct (Sonoda *et al.*, 2013). IL-8 is the chemokine that recruits leukocytes (Rychlik *et al.*, 2014).

As AvBDs are members of the innate immune system, and ILs are immunomodulators, they may both play pivotal roles in the intestinal defense system in young chicks. Crhanova *et al.* (2011) reported that the expression levels of *AvBD1*, 2, 4, and 6 as well as of *IL-1 β* and *IL-8* in the cecum were higher during the first 3 days of life and decreased as of day 4. However, it remains unknown whether changes in AvBDs and cytokine expression occur in other intestinal segments. Cuperus *et al.* (2016) reported that AvBD9 is predominantly expressed in embryonic cells throughout the intestine. They also showed that an AvBD9 immuno-positive area was stable during development, except that the positive area in the jejunum was larger on day 1 post hatching than in embryos or 4-day-old chicks. However, the localization of other AvBDs, and their changes during chick growth, are unknown. The aim of this study was to determine the changes in the expression of *AvBDs* and *IL-1 β* , -6, and -8, and the localization of AvBD2 in the ileum and cecum in broiler embryos and chicks during their growth. We focused on the immunolocalization of AvBD2, because the antibody to AvBD2 was the best antibody that was raised in our laboratory to identify AvBDs in the intestine.

Materials and Methods

Experimental Birds and Tissue Collection

Fertilized eggs and male day-old chicks of broiler (Chunky) were obtained from a local hatchery (Fukuda Poultry, Okayama, Japan). The eggs were incubated in a moisturized incubator for 19 days. Chicks were maintained in a brooding room with a lighting schedule of 23 h light:1 h dark for 7 days. The chicks were given a commercial starter diet (Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) and water *ad libitum*. Embryos at day 19 (ED19), day-old (before feeding; D0) and 7-day-old chicks (D7) were euthanized using carbon dioxide, and their ileums and cecums were collected ($n=5$ each). This study was approved by the Hiroshima University Animal Research Committee (No. C15-16).

RNA Isolation and Reverse Transcription PCR (RT-PCR)

RNA samples were extracted from the ileum and cecum using Sepazol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's directions. RNA was dissolved in 10 mM Tris with 1 mM EDTA (pH 8.0) and stored at -80°C until use.

The RNA was treated with 1 U RQ1 RNase-free DNase (Promega Co, Madison, WI, USA) in a 10- μl reaction mixture (10 μg total RNA, 1 \times DNase buffer and 1 U DNase) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA) programmed at 37°C for 30 min and then at 65°C for 10 min. The reaction was stopped with 1 U RQ1 DNase Stop Solution (Promega Corporation, Madison,

USA). The concentration of RNA was measured using a NanoDrop Lite instrument (Thermo Fisher Scientific, Waltham, WV, USA). The RNA was then reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. The reaction mixture (10 μl) consisted of 0.5 μg total RNA, 1 \times reverse transcription buffer (Toyobo Co. Ltd.), 1 μM deoxyribonucleotide triphosphate (dNTP) mixture (Toyobo Co. Ltd.), 5 U RNase inhibitor (Toyobo Co. Ltd.), 0.25 μg of oligo(dT)20 (Toyobo Co. Ltd.), and 50 U ReverTra Ace. Reverse transcription was carried out at 42°C for 30 min, followed by heat inactivation at 99°C for 5 min, in a programmable thermal controller (PTC-100; MJ Research). The cDNA samples were stored at -20°C until use.

Real-time PCR

Real-time PCR was carried out using the AriaMix Real-time PCR system (Agilent Technologies Japan Ltd., Tokyo, Japan). The reaction mixture (10 μl) consisted of 0.5 μl cDNA, 1 \times Brilliant III SYBR Green QPCR Mix (Agilent Technologies Japan Ltd.), 0.25 μM of each primer, and water. The primers used for PCR are listed in Table 1. Two thermal protocols (two- or three-step) were used for PCR, depending on the target gene. For the two-step protocol, thermal cycles were as follows: 50 cycles of 95°C for 5 s and 62°C (*AvBD2* to 4, 6, 12, *IL-1 β* , -8, and *RPS17*) or 63°C (*AvBD5*, 10, and *IL-6*) for 10 s. For the three-step protocol, we used 50 cycles of 95°C for 5 s, 55°C (*AvBD1* and 7) or 58°C (*AvBD8*), and 72°C for 10 s. Because expression of *AvBD9*, 11, 13, and 14 was not detected by real-time PCR in a preliminary analysis, these genes were not further examined (data not shown). 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 housekeeping gene *RPS17* (Livak and Schmittgen, 2001). An RNA sample of ED19 was used as a standard sample.

Immunohistochemistry

Anti-serum to AvBD2 was raised by immunization of rabbits with keyhole limpet hemocyanin-conjugated synthetic peptide (Medical & Biological LAB, Nagoya, Japan). The sequence of the synthetic peptide was CPSHLIKVGS. The antibody was purified using HiTrapTM NHS-activated HP conjugated with synthetic peptide (GE Healthcare Japan, Tokyo, Japan).

The ileum and cecum were fixed in 10% (v/v) formalin in phosphate-buffered saline (PBS), and processed for paraffin sectioning, at 4 μm thickness. The sections were deparaffinized and then autoclaved at 110°C for 15 min in 10 mM Tris with 1 mM EDTA (pH 10) for antigen retrieval. After cooling and washing with PBS (5 min, 3 times), endogenous peroxidases were quenched by incubation in 0.3% (v/v) H_2O_2 in methanol for 10 min. After washing with PBS (5 min, 3 times), the sections were incubated with 5% (w/v) blocking reagent (Roche Co., Basel, Switzerland) for 1 h to block non-specific binding, followed by incubation with an antibody specific to AvBD2 diluted at 5 $\mu\text{g}/\text{ml}$ in PBS for 16 h. The sections were then washed in PBS (5 min, 3 times), and

Table 1. PCR primers used for profiling of AvBDs and IL-1 β , -6 and -8

Gene	sequences (5' -3')	Accession No. (Reference)
<i>AvBD1</i>	F GATCCTCCCAGGCTCTAGGAAG R GCCCATATTCTTTTGC	NM_204993 (a)
<i>AvBD2</i>	F GTTCTGTAAAGGAGGGTCCTGCCAC R ACTCTACAACACAAAACATATTGC	NM_001201399 (b)
<i>AvBD3</i>	F CCACTCAGTGCAGAATAAGAG R AATTCAGGGCATCAACCTC	NM_204650 (c)
<i>AvBD4</i>	F ATCGTGCTCCTCTTTGTGGCAGTTCA R TACAACCATCTACAGCAAGAATACT	NM_001001610 (b)
<i>AvBD5</i>	F GCTGTCCCTTGCTCGAGGATT R GGAATACCATCGGCTCCGGC	NM_001001611 (b)
<i>AvBD6</i>	F GATCCTTTACCTGCTGCTGTCT R TCCTCACACAGCAAGATTTTAGTC	NM_001001612 (b)
<i>AvBD7</i>	F ACCTGCTGCTGTCTGCTCCTC R TGCACAGCAAGAGCCTATTC	NM_001001194 (a)
<i>AvBD8</i>	F TTCTCCTCACTGTGCTCCAA R AAGGCTCTGGTATGGAGGTG	NM_001001194 (a)
<i>AvBD9</i>	F GCTTACAGCCAAGAAGACGCT R GGAGCTAGGTGCCATTTGCA	NM_001001194 (a)
<i>AvBD10</i>	F TGGGGCACGCAGTCCACAAC R CATGCCCCAGCACGGCAGAA	NM_001001609 (c)
<i>AvBD11</i>	F ACTGCATCCGTTCCAAAGTCTG R TCGGGCAGCTTCTCTACAAC	NM_001001617 (b)
<i>AvBD12</i>	F GGAACCTTTGTTTCGTGTTCA R GAGAATGACGGGTCAAAGC	NM_001001194 (a)
<i>AvBD13</i>	F GATCCTCCAGCTGCTCCTTG R AGTGGCCATGGTTGTTCTC	NM_001001780 (a)
<i>AvBD14</i>	F CATATTCCTCCTGTTTCTTGTCTC R GCCAGTCCATTGTAGCAGGT	NM_001001620 (b)
<i>IL-1β</i>	F ACTGGGCATCAAGGGCTA R GGTAAGAAGATGAAGCGGGTC	NM_204524 (d)
<i>IL-6</i>	F AGAAATCCCTCCTCGCCAAT R AAATAGCGAACGGCCCTCA	NM_204628 (e)
<i>IL-8</i>	F CTGTCCTGGCCCTCCTCTGGTT R TGGCGTCAGCTTCACATCTTG	NM_001167719 (f)
<i>RPS17</i>	F AAGCTGCAGGAGGAGGAGAGG R GGTGGACAGGCTGCCGAAGT	NM_204217 (e)

References for each primer are as follows; a=Xu *et al.* (2015), b=Subedi *et al.* (2007), c=Ebers *et al.* (2009), d=Zang *et al.* (2012), e=Nii *et al.* (2011), f=Sundaresan *et al.* (2007).

incubated with biotin-conjugated anti-rabbit IgG (1:200) and avidin-biotin-peroxidase complex (1:50) for 1 h each using a VECTASTAIN ABC kit (Vector Labs, Inc., Burlingame, CA, USA). The immunoreaction products were visualized using a reaction mixture of 0.02% (w/v) 3, 3'-diaminobenzidine-4HCl and 0.05% (v/v) H₂O₂, and then, the sections were counterstained with hematoxylin.

Negative control staining by replacing the primary AvDB2 antibody with absorbed antibody was used to confirm antibody specificity. The absorbed antibody was prepared by incubating 5 μ g AvBD2 antibody and 25 μ g synthetic AvBD2 peptide in 1 ml of PBS at 4°C for 16 h.

Image Analysis

Sections were examined under a light microscope connected to an image analysis software (NIS-Elements, Nikon, Tokyo, Japan). AvBD2-positive cells in the whole area of

mucosal tissue (mucosal epithelium, crypts, and lamina propria) on each section of the ileum and cecum were counted, followed by calculation of positive cell numbers in an area of $1 \times 10^5 \mu\text{m}^2$.

Statistical Analysis

All data are expressed as the mean \pm SEM. The significance of differences in gene expression levels and the AvBD2-positive cell counts among different growth stages were obtained using one-way ANOVA followed by Tukey's test, or the Kruskal-Wallis test followed by the Steel-Dwass test. Differences were considered significant when the *P* value was <0.05 .

Results

Figure 1 shows the changes in the expression of 10 AvBDs (*AvBD1*–8, 10, and 12) in the ileum and cecum during em-

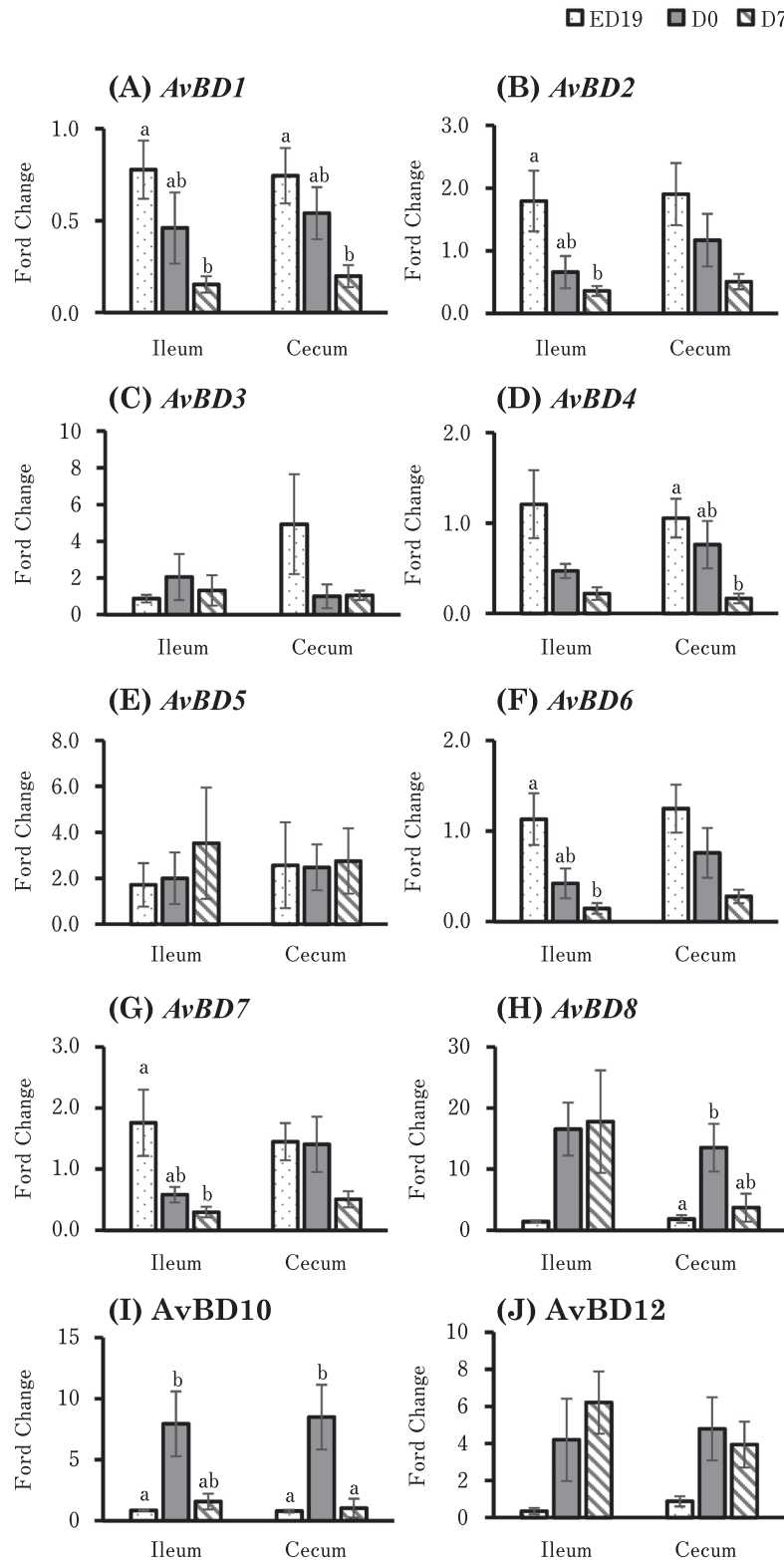


Fig. 1. Changes in the gene expression levels of *AvBD1-8, 10, and 12* in the ileum and cecum during the growth of embryos and chicks. Values are the mean \pm SEM ($n=5$) of fold changes in expression. ^{a,b} Values with different letters are significantly different among ED19, D0 and D7 within the ileum or cecum ($P<0.05$).

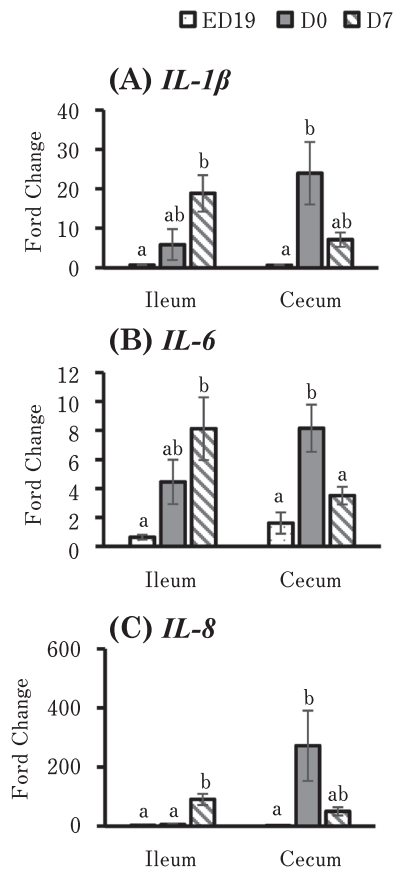


Fig. 2. Changes in the gene expression levels of *IL-1β*, *-6*, and *-8* in the ileum and cecum during the growth of embryos and chicks. The values are the mean \pm SEM ($n = 5$) of fold changes in expression. ^{a,b} Values with different letters are significantly different among ED19, D0 and D7 within the ileum or cecum ($P < 0.05$).

bryo and chick growth. In the ileum, the expression levels of *AvBD1*, 2, 6, and 7 were significantly lower on D7 than on ED19 (Fig. 1A, B, F, and G). In contrast, the expression level of *AvBD10* on D0 was significantly (~7-fold) higher than that on ED19 (Fig. 1I). However, the expression levels of *AvBD3*, 4, 5, 8, and 12 were not significantly different among ED19, D0, and D7 (Fig. 1C–E, H, and J). In the cecum, the expression levels of *AvBD1* and 4 on D7 were significantly lower than those at ED19 (Fig. 1A and D). The expression levels of *AvBD8* and 10 in the cecum were significantly higher on D0 than on ED19, and that of *AvBD10* was decreased on D7 (Fig. 1H and I). However, the expression levels of *AvBD2*, 3, 5, 6, 7, and 12 did not significantly change during growth from ED19 to D7 (Fig. 1B, C, E, F, and G).

Figure 2 shows the changes in the expression of *IL-1β*, *-6*, and *-8* in the ileum and cecum during growth. The expression levels of *IL-1β*, *-6*, and *-8* in the ileum on D7 were approximately 16-, 7-, and 90-fold higher, respectively, than those on ED19 (Fig. 2A to C). The expression levels of *IL-*

1β, *-6*, and *-8* in the cecum were approximately 23-, 5-, and 270-fold higher on D0 than on ED19, and expression of *IL-1β* and *-6* declined on D7 (Fig. 2A to C).

Figure 3 shows the localization of AvBD2-positive cells in the ileum and cecum on ED19, D0, and D7. The AvBD2-positive cells were localized in the lamina propria, beneath the epithelium of the villi and crypts in both the ileum (Fig. 3A, C, E) and the cecum (Fig. 3B, D, F) at all stages. A few positive cells were localized in the epithelium of villi and crypts (Fig. 3D). The localization profiles of AvBD2 in the ileum and cecum were not different among the growth stages evaluated. Figure 4 shows the frequencies of AvBD2-positive cells (cell number in an area of $1 \times 10^5 \mu\text{m}^2$) in mucosal tissues. In the cecum, the AvBD2-positive cell frequency was significantly higher on D0 than on ED19 or D7. In contrast, their frequency in the ileum was not significantly different among the growth stages. Negative control staining using absorbed antibody did not reveal any reaction products in either the ileum or the cecum (Fig. 3G, H).

Discussion

We report the changes in the expression levels of AvBD genes and *IL-1β*, *-6*, and *-8* and the localization of immunoreactive AvBD2 in the ileum and cecum of chicken embryos and chicks during their early phase of growth, i.e., ED19, D0, and D7. Major findings were that (1) AvBD expression levels showed 3 distinct profiles: highest on D0 (*AvBD8* and 10), decreased after hatching (*AvBD1*, 2, 4, 6, and 7), and no changes (*AvBD3*, 5, and 12) in the ileum or cecum; (2) the *IL-1β*, *-6* and *-8* expression levels were increased on D7 in the ileum and on D0 in the cecum; and (3) the AvBD2-positive cells were localized in the lamina propria of the ileum and cecum, and their frequency in the cecum was higher on D0 than on ED19 or on D7.

The results of the current study showed that expression levels of *AvBD1*, 2, 6, and 7 in the ileum, and of *AvBD1* and 4 in the cecum on D7 were decreased as compared to the levels on ED19. The highest expression levels of *AvBD8* and 10 were also found in the cecum on D0, and that of *AvBD10* declined by D7. These results, showing decreased AvBD expression with post-hatch growth, support the findings reported by Crhanova *et al.* (2011) that in the first three days of life, the chicken cecum was protected by increased expression of *AvBD1*, 2, 4, and 6, the expression of which dropped as of day 4 of life. The current study showed that the decline in AvBD expression in the post-hatch period occurs not only in the cecum, but also in the ileum. Su *et al.* (2017) reported that *Eimeria acervulina* challenge resulted in the downregulation of *AvBD1*, 6, 10, 11, 12, and 13 in the duodenum, and that *Eimeria maxima* challenge downregulated *AvBD6*, 10, and 11 in the duodenum and *AvBD10* in the jejunum. The complexity of microbiota gradually increases in the chick intestine, with the most dramatic development occurring during the first week of life (Crchanova *et al.*, 2011). Accordingly, as observed in the current study, the expression of multiple AvBDs changed during the first week of life, and there seems to be an association between the

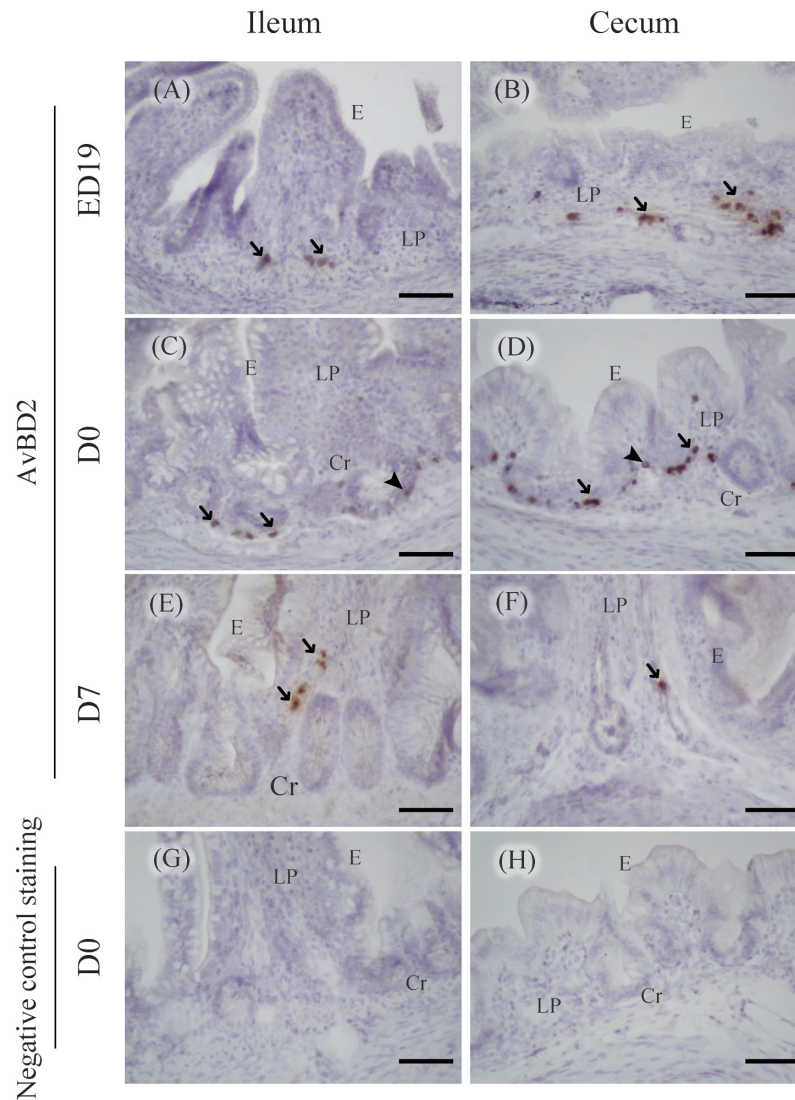


Fig. 3. Micrographs showing immunoreactive AvBD2 in the ileum and cecum on embryonic day 19 (ED19), and days 0 (D0) and 7 (D7) of the post-hatching period. AvBD2-immunoreactive cells are localized in the lamina propria beneath the epithelium in each tissue at all developmental stages (arrows). Some of the positive cells are localized in the epithelium of the villi and crypts (arrowheads). G and H show negative control staining using absorbed antibody. E=mucosal epithelium, LP=lamina propria, Cr=crypt. Scale bar=50 μ m.

colonization of natural or specific microbiota complexes and changes in AvBDs expressed during the early post-hatch period. However, challenge with specific microorganisms may cause an upregulation of AvBDs, as expression of *AvBD8* and *13* in the cecum of 21-day-old broiler chickens was upregulated by *Eimeria maxima* (Su *et al.*, 2017), and expression of *AvBD1*, 2, 4, and 6 was increased in the cecal tonsils of broiler chicks 3–5 days post infection by *Salmonella* Typhimurium, starting from 1 day of age (Akbari *et al.*, 2008). The intestinal microbiota composition plays an im-

portant role in the maintenance of gut health (Clavijo and Flórez, 2018). It is reported that AvBD1 is cytotoxic to *E. coli* and *E. faecalis*, and AvBD4 and 10 are cytotoxic to *S. Typhimurium* and *E. coli* (Yacoub *et al.*, 2015). Lee *et al.* (2016) suggested that *E. coli* is killed by AvBD1–4, 6, 7, 9, 11, 13, and 14. Thus, AvBDs synthesized in the ileum and cecum may play roles in the regulation of the microbiota population.

Our results showed that the expression of *IL-1 β* , -6, and -8 was higher on D0 and D7 than on ED19 in the ileum and

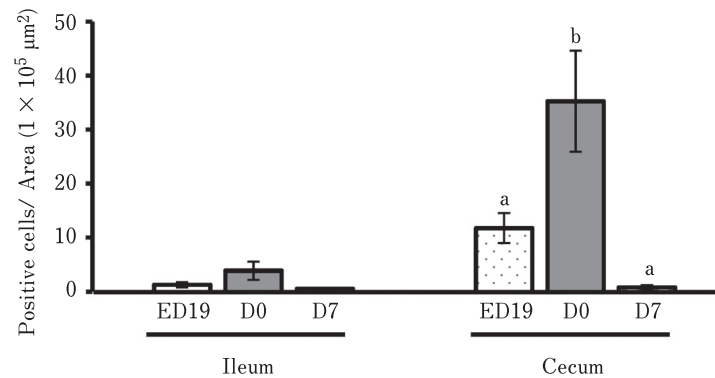


Fig. 4. Changes in the frequencies of AvBD2-positive cells in the mucosa of the ileum and cecum on embryonic day 19 (ED19), and days 0 (D0) and 7 (D7) of the post-hatching period. Values are the mean \pm SEM ($n=5$) of the number of positive cells in an area of $1 \times 10^5 \mu\text{m}^2$. ^{a,b} Values with different letters indicate significantly different within the cecum ($P<0.05$).

cecum, respectively. Thus, the expression of these cytokines may be upregulated in association with the development of intestinal microflora. Crhanova *et al.* (2011) demonstrated that, for the first three days of life, the chicken cecum is protected by AvBDs, and this type of cecum protection was replaced from day 4 of life by normalization of the gut immune system developed by proinflammatory cytokines IL-8 and IL-17. Although we have not examined the expression of IL-17, the higher levels of IL-1 β , IL-6, and IL-8 may suggest that the development of the mucosal immune system may be induced by these cytokines during the first week of life.

Our results showed that AvBD2-positive cells were localized in the lamina propria beneath the epithelium of villi and crypts. Cuperus *et al.* (2016) reported that AvBD9 was predominantly found in the enteroendocrine cells of the intestines. However, expression of AvBD1, 2, and 7 has been identified in the bone marrow tissue in chickens (Derache *et al.*, 2009), suggesting that bone marrow-derived leukocytes are involved in synthesis of these AvBDs. Thus, the AvBD2-positive cells identified in this study may be the member of leukocytes derived from bone-marrow. The frequency of AvBD2-positive cells was increased on D0 compared to ED19 and was decreased on D7 in the cecum. These cells may play an important role in host defense at an early phase of the post-hatch period. Although AvBD2 gene expression was higher on ED19, AvBD protein synthesis might be immature before hatching. The expression of the IL-8 chemokine was also higher on D0. We assume that IL-8 may be one of the factors involved in the recruitment of AvBD2-positive cells in the cecum. Such cells may decrease in association with the development of the gut-acquired immune system. The frequency of AvBD2-positive cells in the ileum was relatively low and remained unchanged during the embryonic and post-hatch phases. The recruitment level of those cells may be unchallenged under healthy conditions

in this particular tissue. The cecum retains intestinal internal contents for the longest period within the intestine, and microbiota of the cecum play roles in the digestion of substances rich in cellulose and others (Clavijo and Flórez, 2018). These differential characteristics in terms of microbiota and intestinal internal contents in the cecum may be considered when examining why changes in the AvBD2 cell population are different between the ileum and cecum.

In conclusion, we suggest that AvBDs are expressed in the ileum and cecum of the embryo and young chick, with levels being high before or right after hatching, and decreasing by D7. AvBD2-positive cells are likely recruited in the cecum around the hatching phase. The expression of proinflammatory cytokines increases with the growth of chicks till D7 in the ileum, but showed the highest levels around hatching in the cecum. These AvBDs and proinflammatory cytokines may play roles in the host defense in the intestinal mucosa of chick embryos and neonates.

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