Effects of Probiotics on the Expression and Localization of Avian \( \beta \)-defensins in the Proventriculus of Broiler Chicks

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The aim of this study was to determine the effects of probiotics-feeding on the gene expression and protein localization of avian \( \beta \)-defensins (AvBDs) in the proventriculus of broiler chicks. Male broiler chicks were arranged in 3 groups: control group, probiotics group I and probiotics group II, which were fed with starter rations containing 0%, 0.2% or 0.4% probiotics, respectively, from day 0 (D0; at one day old) to D14. Proventriculi in all groups were collected at D0, D7 and D14 for analysis of AvBDs expression and AvBD12 protein localization. The expression of AvBDs genes was examined by reverse transcription-PCR and changes in the expression upon probiotics-feeding were examined by real-time PCR. The AvBD12 localization was examined by immunohistochemistry, and density of immunoreactive products was examined by image analysis under a microscope. Out of 14 AvBDs genes, seven AvBDs were detected in the proventriculus of chicks, namely, AvBD1, 2, 4, 6, 7, 10 and 12. The expression of the 7 detected genes did not show any significant differences between control and probiotics groups at D7 and D14. The immunoreactive (ir) -AvBD12 was localized in surface epithelium and cells in the connective tissues of proventricular glands. The ir-AvBD12 density in surface epithelium was significantly higher at D7 than at D0 or D14 in control group. At D7 and D14, the ir-AvBD12 density was significantly lower in probiotics groups than in control group. The ir-AvBD12 cells in proventricular gland increased in number with age; however, there were no significant differences between control and probiotics groups at D7 and D14. These results suggest that, although probiotics-feeding does not affect the gene expression of AvBDs, it may induce AvBD12 secretion from the surface epithelium of the proventriculus in broiler chicks.

Key words: avian \( \beta \)-defensins, chick proventriculus, innate immunity, probiotics

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Introduction

The digestive tract is the organ mainly infected by pathogenic agents in feed. Innate immunity may play roles in the protection against infection in newly hatched chicks because gut-associated lymphoid tissues have not fully matured during the first few weeks of life (Miyazaki \textit{et al.}, 2007). The innate immune response occurs at early phase of infection and attack pathogens less specifically than adaptive immune response (Boyd \textit{et al.}, 2007; Werling and Coffey, 2007). Innate immune responses are stimulated by pathogen recognition receptors such as Toll-like receptors. The stimulation of these receptors may activate many cellular pathways, resulting in the expression of cytokines and antimicrobial peptides (Brogden \textit{et al.}, 2003; Lemaitre \textit{et al.}, 1997). Avian \( \beta \)-defensins (AvBDs) are antimicrobial peptides that play important roles in the innate immunity of avian species. Defensins play essential roles as a first line of defense against invading pathogens via their anti-microbial activity by breaking down the bacterial cell membrane (Sahl \textit{et al.}, 2005). Some defensins may also act as chemoattractants for monocytes, lymphocytes and dendritic cells, which is considered as a link between innate and adaptive immune responses (Yang \textit{et al.}, 1999; Ganz, 2003). To date, a total of 14 AvBD genes (AvBD1 to 14) have been identified in different tissues of the chicken (Lynn \textit{et al.}, 2004, 2007; Xiao \textit{et al.}, 2004).

Recently, it was reported that probiotics-feeding decreased the colonization of Salmonella and Campylobacter bacteria in the intestines (Haghighi \textit{et al.}, 2008; Ghareeb \textit{et al.}, 2012). Haghighi \textit{et al.} (2005, 2006) reported that probiotics-feeding induced natural antibodies in unimmunized chickens, and enhanced antibody responses to antigens. Probiotics

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also affected the expression of cytokines such as IL-12 and IFN-γ, and the density of IL-6-expressing cells and CD8+ T cells in the intestinal mucosa (Haghighi et al., 2008; Yoshimura et al., 2010; Huang et al., 2013). The expression of human β-defensin 2 in intestinal epithelial cells was shown to be upregulated by a number of probiotic bacteria including Lactobacillus (Wehkamp et al., 2004; Schlee et al., 2008). Thus, it is expected that probiotics-feeding may affect the innate immune functions in the digestive system of chicks, and it would be beneficial if such effects appear in the chicks before their adaptive immunity develops. Previous studies showed that AvBDs were expressed in the gastrointestinal mucosa including proventriculus in chicks, and that probiotics down-regulated the expression of AvBDs that was elevated by Salmonella infection in the cecum, where the bacterial colonization is high (Akbari et al., 2008; Rodríguez-Lecompte et al., 2012). However, there are no reports that examined the effects of probiotic bacteria on the localization of AvBD proteins in the proventriculus of chicks.

The aim of this study was thus to determine the effects of feeding of probiotics, consisting of Streptococcus faecalis, Clostridium buthricum and Bacillus mesentericus, on the gene expression of AvBDs and localization of AvBD12 protein in the proventriculus of broiler chickens. The proventriculus was focused on because undigested feed that may contain many microorganisms enters, but there is little information for the effects of probiotics on the defense system mediated by AvBDs in this segment. Immunolocalization was focused on AvBD12 because among the antibodies to several different AvBDs that had been raised by us, only the AvBD12 antibody showed the specific immunoreactivity on the proventriculus section in this study.

Materials and Methods

Treatments of Birds and Tissue Collection

One-day-old male broiler chicks (Chunky broilers) were purchased from a local hatchery (Fukuda Poultry, Okayama, Japan). They were divided into 3 groups, namely, control group, probiotics group I and probiotics group II. Chicks in the control group were given only commercial starter diet (Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan), whereas chicks in probiotics groups I and II were given the starter rations containing probiotics (Toaraze for chickens, Toa Pharmaceutical Co. Ltd., Tokyo) at concentrations of 0.2% and 0.4% (wt/wt), respectively. The Toaraze for chickens contained Streptococcus faecalis (>1×10^6/g), Clostridium buthricum (>1×10^7/g) and Bacillus mesentericus (>1×10^7/g). Chicks were maintained in a brooding room under lighting conditions of 23 h light/1 h dark for 7 days, followed by 20 h light/4 h dark until 14 days of age. The birds were reared under ad libitum access to feed with or without probiotics and water. On days 0 (at one day old), 7 and 14 (D0, D7 and D14), they were euthanized using carbon dioxide and the proventriculi were collected (n=6 in all groups on each day). Part of the collected tissue was used for AvBDs gene expression analysis and the other part was used for AvBD12 immunohistochemistry. Birds were handled in accordance with the regulations of Hiroshima University Animal Research Committee.

Reverse Transcription (RT)-PCR Analysis

For cDNA preparation, total RNA was extracted from the mucosal tissues of the proventriculus using Sepazol RNA I super according to the manufacturer’s directions (Nacalai Tesque, Kyoto, Japan). The obtained RNA pellet was then dissolved in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA) and kept at −80°C until use. The concentration of the total RNA was determined using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK) in each sample. The samples were treated with 1 U RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA), programmed at 37°C for 45 min and 65°C for 10 min. The concentration of RNA in each sample was measured after DNase using Gene Quant Pro (Amersham Pharmacia Biotech). Then, the RNA samples were reverse-transcribed using Rever Tra Ace (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer’s instructions. The reaction mixture (10 μl) consisted of 1 μg of total RNA, 1x Reverse Transcription buffer, 1 μM deoxynucleotide triphosphate mixture, 20 U RNase inhibitor, 0.5 μg of oligo (dT) 20 and 50 U Rever Tra Ace. The reverse transcription was performed at 42°C for 30 min, followed by heat inactivation for 5 min at 99°C using a programmable thermal controller (PTC-100; MJ Research).

Semi-quantitative and Real-time Polymerase Chain Reaction (PCR) Analysis

The PCR analysis was performed to identify the AvBDs expressed in the proventriculus using samples of D7 chicks (n=6) in the control group. Reaction mixture (25 μl) containing 0.5 μl of cDNA, 1X PCR buffer, 0.2 μM dNTP mixture, 0.5 μM of each primer (forward and reverse) and 0.125 U Takara Taq (Takara Bio Inc., Shiga, Japan) was prepared. Table 1 shows the AvBDs primers used for PCR. The PCR cycle parameters were 35 cycles (for AvBD1, 2, 4, 6, 7, 12 and RPS17) or 40 cycles (for AvBD10) of denaturation at 94°C for 30 sec, annealing at 60°C (for AvBD1, 2, 4, 6, 7 and 12) or 57°C (for AvBD10 and RPS17) for 30 sec and extension at 72°C for 1 min, followed by final extension at 72°C for 6 min. The PCR products were separated by electrophoresis on 2% (wt/vol) agarose gels containing 0.25% (wt/vol) ethidium bromide and photographed under UV illumination.

The expression level of the AvBDs detected by RT-PCR analysis (AvBD1, 2, 4, 6, 7, 10 and 12) was further analyzed by real-time PCR using the Roche Light Cycler Nano system (Roche Applied Science, Indianapolis, IN). The reaction mixture (20 μl) containing 1 μl of cDNA, 10 μl of Thunder Bird SYBR qPCR Mix (Toyobo Co. Ltd., Osaka, Japan), 1 μM of each primer, and 0.4 μl of Rox reference dye and Milli Q water were taken into PCR tubes (Roche Diagnostics GmbH, Mannheim, Germany). The thermal protocols for PCR were 50 cycles at 95°C for 10 sec; and 60°C (AvBD1 and RPS17), 61°C (AvBD2), 62°C (AvBD12) or 63°C (AvBD4, 6, 7 and 10) for 30 sec. Real-time PCR data were analyzed using the 2^ΔΔCt method to calculate the relative
level of \textit{AvBDs} expression in each sample and were expressed as ratios in relation to the \textit{RPS17} housekeeping gene (Livak and Schmittgen, 2001). An RNA sample of a D0 chick was used for a standard sample.

**Histology and Immunohistochemistry**

Localization of \textit{AvBD12} was examined by immunohistochemistry using rabbit anti-\textit{AvBD12} antibody that was available in our laboratory. The anti-\textit{AvBD12} antibody had been raised in rabbit and purified using a Hi Trap affinity protein G HP column (GE Health Bio-Sciences AB, Uppsala, Sweden) and was used in our previous studies (Abdel Mageed \textit{et al}., 2009; Abdelsalam \textit{et al}., 2010). The tissues were fixed with 10\% (vol/vol) formalin in phosphate-buffered saline (PBS), dehydrated, embedded in paraffin wax and sliced into sections of 4\,\mu m thickness for histological and immunohistochemical examinations. Antigen retrieval was performed by autoclaving the sections for 1 min in 0.1\,M citric acid, pH 6.0. Immunohistochemistry was performed using Vecta Stain ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were incubated with blocking solution, 1.5\% (vol/vol) normal goat serum in PBS, for 30\,min. Then, they were incubated overnight with rabbit anti-\textit{AvBD12} antibody diluted at a concentration of 20\,\mu g/ml in PBS, followed by washing with PBS (3\times5\,min). The sections were incubated with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex for 1\,h each, and were washed with PBS (3\times5\,min) after each step. Immunoprecipitates were visualized by incubating the sections with a mixture of 0.02\% (wt/vol) diaminobenzidine (Sigma, St. Louis, MO) and 0.001\% \textit{H}_2\textit{O}_2 (vol/vol) in 0.05\,M Tris-HCl (pH 7.6). The sections were dehydrated and covered. For the control staining, the primary antibody of \textit{AvBD12} was replaced with normal rabbit IgG (20\,\mu g/ml) or preabsorbed \textit{AvBD12} antibody that was prepared by incubating the

| Gene   | Sequence of forward and reverse primers | Accession No. (references) | Expected product size (bp) |
|--------|-----------------------------------------|----------------------------|---------------------------|
| \textit{AvBD 1} | F-AAACCATGCGGATCTGTAATCTGC R-CAATGCTAAACTGCAACCTTTA | (a) AF03335 | 405 |
| \textit{AvBD 2} | F-GTCTTGTAAAGGAGGTCCTGCCAC R-ACTCTACAACACACCATATGCC | (a) AF03336 | 238 |
| \textit{AvBD 3} | F-CTGCCCGGCTCCCACACATAG R-GCAATGCAAACCTCAGCAACCTTTA | (a) NM_204650 | 275 |
| \textit{AvBD 4} | F-ATCGTGCTCCTCTTTGTGGCAGTTCA R-CTACAACACCCTCTACAGAAATAT | (a) NM_00101610 | 171 |
| \textit{AvBD 5} | F-ATGCGAGATCCTGTCGCTCTTCTCTTTTC T-CAGGAAATACCATCCTGCCGCAGCAAGAA | (a) NM_00101608 | 201 |
| \textit{AvBD 6} | F-GATCTTCTTATACGTGCTGTCT T-CCTCAACAGCAAGATTATTAGT | (a) NM_0010193 | 184 |
| \textit{AvBD 7} | F-CTGCTGTTGCTTCTCTTTTGTGG C-ATTTGTGTAATGCGAAGGAAGA | (a) NM_0010194 | 230 |
| \textit{AvBD 8} | F-ACAGTGTTGACAGCAGGAGGGGA R-CTCTCTGCTGCTCAGCTTTTGTTGA | (a) NM_00101781 | 123 |
| \textit{AvBD 9} | F-ATGAGAAATCTCTTTCTCTTCTGTGTC R-CTAGGAGCTAGGTGACAATGGCTCCCATTTTGTCAGCA | (a) NM_00101611 | 204 |
| \textit{AvBD 10} | F-TGGGGCAGTCACCAAC C-ATGCCCCGCAAGGGAGAA | (b) NM_00101609 | 157 |
| \textit{AvBD 11} | F-CTGCAGCCCTTCCAACAGCTTCTG CT-CGGGCCGAGCCGAAGAAGAGAAT | (a) NM_00101779 | 301 |
| \textit{AvBD 12} | F-CCCCAGCAGGACAAAGCAGGATG R-AGTAAGTACACGAGTATTC | (c) NM_00101607 | 157 |
| \textit{AvBD 13} | F-CTATGTGTCAGTCATCTCTTCTGCAATC R-CTCTGAGCTGTTGACG | (a) NM_00101780 | 270 |
| \textit{AvBD 14} | F-CATATTCCTCCTCTCTCTCAGR-GCCATCTCCAGTCTACG | (d) AM402954 | 150 |
| \textit{RPS 17} | F-AGAATCTGGACAGAGGAGGAGGAGG R-GTGTTGAGCAGGCTCCAGAAGT | (c) NM_0024217 | 136 |

(a) Abdel Mageed \textit{et al}., 2008, (b) Ebers \textit{et al}., 2009, (c) Upadhyaya \textit{et al}., 2013, (d) Meade \textit{et al}., 2009, (e) Ariyadi \textit{et al}., 2012.)
antibody with AvBD12 peptides used for immunization at a ratio 1:4 (AvBD12 antibody: AvBD12 peptides) overnight at 4°C.

**Image Analysis**

The density of AvBD12 immunoreaction products (immunoreactive (ir)-AvBD12) in the sections of the proventriculus of each group was examined under a light microscope with image analysis software (NIS-Elements, Nikon, Tokyo, Japan). For analysis of the density in the surface epithelium, the immunopositive area and the total measured area were analyzed, and the ratio of positive area to the total measured area was obtained. In glandular tissue, the numbers of AvBD12-immunopositive cells (ir-AvBD12 cells) in glandular lobes tissues, excluding the lumen spaces, were counted. Three different regions in one tissue were measured in each analysis and the mean value was used for the value of one tissue.

**Statistical Analysis**

The significance of differences in AvBD expression levels, the density of ir-AvBD12 in the surface epithelium and the counts of ir-AvBD12 cells in the glandular lobes among the different ages within control and probiotics groups and among different groups at D7 and D14 was analyzed by Tukey’s test. Differences were considered significant when the P value was <0.05.

**Results**

The inner mucosa of the proventriculus formed well-developed folds lined by surface epithelium of columnar cells. The deep layer of mucosa contained proventricular gland lobes. No histological abnormalities or inflammatory features were observed in the proventricular tissue of all groups (data not shown). In terms of the profile of AvBD expression in the mucosa of the proventriculus of D7 control group chicks, seven AvBD genes were expressed, namely, AvBD1, 2, 4, 6, 7, 10 and 12 (Fig. 1). Figure 2 shows the effects of age and probiotics-feeding on the expression of those AvBDs in the proventriculus at D0, D7 and D14. The expression levels of the 7 AvBDs in the control and probiotics groups did not show significant differences with age and probiotics-feeding.

Figures 3 and 4 show the localization of ir-AvBD12 in the proventriculus of chicks in control and probiotics groups at D7 and D14. The ir-AvBD12 was identified in many of the surface epithelial cells in the control group (Fig. 3a, b); however, it was scattered in the surface epithelium in probiotics groups I (Fig. 3c, d) and II (Fig. 3e, f) at D7 and D14. The ir-AvBD12 cells were also localized in the proventricular glands (Fig. 4). They were numerous in the central part of the glandular lobes compared with the peripheral parts in the control and probiotics groups at D0 (data not shown), and D7 and D14 (Fig. 4a–f). The ir-AvBD12 in the surface epithelial cells was identified in their cytoplasm (Fig. 5a), while ir-AvBD12 cells in the proventricular glands were localized in the connective tissue of glandular lobes (Fig. 5b). Negative control staining using preabsorbed AvBD12 antibody (Fig. 5c, d) or normal rabbit IgG (data not shown) did not show positive staining products in either surface epithelium or proventricular glands.

The effects of age and probiotics-feeding on the density of ir-AvBD12 in the surface epithelium and ir-AvBD12 cells in the proventricular glands are shown in Fig. 6. In the surface epithelium of the control group, the ir-AvBD12 density was significantly increased at D7 compared with that at D0 (P<0.05), and then returned to a level similar to that at D0 at D14. The ir-AvBD12 density in probiotics groups I and II was significantly lower at D7 and D14 than in the control (P<0.01). At D7, the density of ir-AvBD12 in group II was significantly lower (P<0.01) than that in probiotics group I, while at D14, the differences between the probiotics groups were not significant (Fig. 6a).

The differences in the frequency of ir-AvBD12 cells in the connective tissue of the proventricular glands among different ages and probiotics groups are shown in Fig. 6b. The frequency of the cells significantly increased at D7 and D14 compared with that at D0 in control and probiotics groups I and II. However, differences in their frequency among the control and probiotics groups were not significant at D7 or D14.

**Discussion**

We here report the effects of probiotics-feeding on the expression of AvBDs and AvBD12 protein localization in the chick proventriculus. The major findings of this study are as follows: (1) out of 14 AvBDs genes, seven genes were detectable in the proventricular mucosal tissue (AvBD1, 2, 4,
6, 7, 10 and 12); (2) ir-AvBD12 was localized in the surface epithelium and cells in the connective tissue of glandular lobes; and (3) the density of ir-AvBD12 in the surface epithelium was decreased by probiotics-feeding at D7 and D14 compared with that in the control. Rodríguez-Lecompte et al. (2012) identified the expression of AvBD3, but not AvBD6, in the proventriculus and intestinal tissues of Ross-308 chicks. However, the current study using Chunky broiler chicks, we identified the expression of AvBD6, but not AvBD3, in the proventriculus of Chunky broiler chicks.

It is thus likely that there are differences in the AvBDs expressed in the proventriculus in different breeds of chicks.

In this study, the ir-AvBD12 was localized in the surface epithelium and cells in the proventricular glands. The ir-AvBD12 identified in the surface epithelium may be secreted into the proventricular lumen to kill bacteria in the luminal contents. The secretion of AvBDs synthesized by the surface epithelium has also been reported in the uterus of hen (Abdel Mageed et al., 2009). The type of ir-AvBD12 cells in the proventricular glands has not been established in this study.

Fig. 2. Effects of probiotics-feeding on the mRNA expression of avian β-defensins (AvBD1, 2, 4, 6, 7, 10 and 12) in chick proventriculus. Values are mean±S.E. of fold changes in expression (n=6). Control groups, and probiotics groups I and II were fed 0%, 0.2% and 0.4% probiotics, respectively. D0, D7 and D14 refer to the probiotics treatment days (D0=one-day-old chicks).
Isobe et al. (2011) identified lingual antimicrobial peptides, a member of the β-defensin family, in the chief cells of abomasum of calves. However, the ir-AvBD12 cells in the proventricular gland seem to be members of the leukocytes, not glandular cells, because they were localized in the glandular lobe connective tissue. Although it is unknown whether synthesized AvBD12 is secreted in the gastric juice, it may attack pathogenic microbes if they infect this tissue. The AvBDs are expressed in various organs in chickens (Cuperus et al., 2013), and may play roles in the mucosal defense system in combination with other immune system components such as leukocytes, cytokines and immunoglobulins (Goto and Kiyono, 2012; Kinnebrew and Pamer, 2012). We assume that the AvBDs synthesized in the proventricular mucosa also play roles in forming the mucosal barrier against invading bacteria.

In the control group, there were no significant differences in the expression level of AvBD12 among D0, D7 and D14. However, the densities of ir-AvBD12 in the surface epithelium and the ir-AvBD12 cells in the proventricular gland lobes were greater in D7 than in D0. These results suggest that the synthesized AvBD12 accumulated in the surface epithelium, and influx or proliferation of ir-AvBD12 cells occurred to an extent that was not accompanied by significant changes in the gene expression level during this stage. The density of ir-AvBD in the surface epithelium was decreased at D14 compared with that at D7. This result may partially support the report of Crhanova et al. (2011) who described that the neonatal chick cecum was protected by increased expression of AvBDs (AvBD1, 2, 4 and 6), which dropped from day 4 of life.

The gene expression of AvBDs was not affected by pro-
biotics-feeding at D7 and D14. In contrast, the density of ir-AvBD12 was reduced in the surface epithelium by 0.2% or 0.4% probiotics-feeding at D7 and D14, although the frequency of ir-AvBD12 cells in the glandular lobes did not show any significant difference between control and probiotics groups. These results suggest that probiotics may not have a significant effect on AvBD12 expression, but it is possible that the secretion of AvBD12 by the surface epithelium is stimulated by probiotics.

Infection by pathogenic agents such as *Salmonella* and *Campylobacter* has been shown to increase the expression of chemokines, proinflammatory cytokines (Withanage et al., 2004; Carvajal et al., 2008) and cellular immunoresponse, particularly Th1 response (Berndt et al., 2007; Noujaim et al., 2008). In addition, infection by *Salmonella* may also up-regulate the AvBDs expression in chick cecal tissue (Akbari et al., 2008; Wigley, 2013). There are reports that Lactobacillus-based probiotics reduced *Salmonella* infection in neonatal chicks (Akbari et al., 2008; Higgins et al., 2008). Akbari et al. (2008) also showed that probiotics-feeding did not affect the expression of AvBD1, 2, 4 and 6 in the cecum of chicks, as the results of current study showing that AvBDs expression in the proventriculus was not significantly changed. The results of the current study suggest that feeding of probiotics may stimulate AvBD12 secretion of the surface epithelium, as discussed above. The secreted AvBD12 in response to probiotics may play roles to reduce pathogenic agents in the contents of the proventriculus. Akbari et al. (2008) also showed that probiotics feeding downregulated the elevation of AvBDs expression by *Salmonella* in chicks, which may be due to suppression of the expression by probiotics in combination with *Salmonella* infection, or due to a reduction in *Salmonella* load in the intestine. Further examinations on the changes in AvBDs expression in response
to the challenge with pathogens in combination with probiotics are necessary to determine the more precise effects of probiotics on AvBDs synthesis and secretion in the proventriculus.

In conclusion, the current results suggest that AvBDs are expressed in chick proventriculus. Although probiotics-feeding may not significantly affect AvBDs gene expression, that feeding may enhance the release of AvBD12 from surface epithelial cells. The secreted AvBD12 may then play roles in reducing the risk of infection by pathogenic microbes in the contents of the digestive tract.

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Fig. 6. Effects of probiotics-feeding on the density of immunoreactive avian β-defensin 12 in the proventriculus of chicks. (a) Density of immunoreactive AvBD12 in the surface epithelium. Values are mean ± S.E. of the density (ratio of immunostained area to total measured area, n=6). (b) Frequency of AvBD12-immunopositive cells in the proventricular gland. Values are mean ± S.E. of the positive cell frequency (cells in $1 \times 10^6 \mu m^2$, n=6). a-c, m-n, x-y: Values are significantly different among treatment days (D0, D7, D14) within each group ($P<0.05$, Tukey’s test). *, ** Values are significantly different ($P<0.05$, $P<0.01$, Tukey’s test). NS = not significant.
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