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Effects of Probiotics *Lactobacillus reuteri* and *Clostridium butyricum* on the Expression of Toll-like Receptors, Pro- and Anti-inflammatory Cytokines, and Antimicrobial Peptides in Broiler Chick Intestine

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The aim of this study was to determine the effects of live probiotics *Lactobacillus reuteri* (LR) and *Clostridium butyricum* (CB) on the expression of genes of innate immune system in broiler chick ileum and cecum. Chicks were administered 500 μl water with or without LR or CB, daily from day 1 to 6 after hatching. The ileum and cecum were collected on day 7 for analysis of gene expression of Toll-like receptors (TLRs), pro- and anti-inflammatory cytokines, and antimicrobial peptides (AMPs) using real-time PCR. The expression of *TLR2-1* was upregulated by CB in the ileum and that of *TLR5* was upregulated by both LR and CB. Expression of *IL-1β* and *TGFβ2* in the ileum and of *TGFβ3* and *TGFβ4* in the cecum was upregulated by both LR and CB. The gene expressions of avian β-defensin (*AvBD*) *1* and cathelicidin (*CATH*) *3* were upregulated by CB and that of *AvBD4* was upregulated by LR in the cecum. However, the expression of *CATH2* in the ileum was downregulated by LR. These results suggest that probiotic LR and CB treatments affect a part of the innate defense system in the ileum and cecum by modulating the expression of innate immune molecules including TLRs, pro- and anti-inflammatory cytokines, and AMPs.

Key words: antimicrobial peptides, chick intestine, cytokines, innate immunity, probiotics, Toll-like receptor

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Introduction

Infection by pathogenic microorganisms in the chick gut may cause health impairments and bacterial contamination of their products. The gut-associated lymphoid tissues and lymphocyte functions undergo development during the first few weeks of life (Bar-shira *et al*., 2003). The innate immune system and maternal antibodies are important in the protection of chicks from infections by microorganisms before the development of lymphoid tissues.

Toll-like receptors (TLRs) recognize microbe-associated molecular patterns (MAMPs) of bacteria and viruses. Ten TLRs have been identified in chickens. The peptidoglycans and lipoproteins of gram-positive bacteria are recognized by TLR2 that form heterodimers with TLR1 (Keestra *et al*., 2007). Lipopolysaccharides (LPS) of gram-negative bacteria are recognized by TLR4 (St. Paul *et al*., 2013). The bacterial flagellin is recognized by TLR5 (St. Paul *et al*., 2013), and bacterial proteases and heat-stable secretory substances are recognized by TLR15 (de Zoete *et al*., 2011). The ds- and ss-RNA viruses are recognized by TLR3 and 7, respectively (Brownlie and Allan, 2011). TLR21 recognizes unmethylated CpG-oligodeoxynucleotides (ODN) of bacteria and viruses (Keestra *et al*., 2010). The expression of TLR 1–5, 7, 15, and 21 in the different intestinal segments has been revealed in chicks (Mackinnon *et al*., 2009), and the stimulation of TLR ligands modulated the expression of innate immune factors, namely antimicrobial peptides and cytokines in the chick intestine (Terada *et al*., 2020).

Defensins and cathelicidins involved in innate immunity are antimicrobial peptides (AMPs) that exert a broad spectrum of antimicrobial activities against gram-negative and gram-positive bacteria, enveloped viruses, and fungi (Cuperus *et al*., 2013). Fourteen avian β-defensins (AvBD1–14) and four cathelicidins (CATH1–3, CATH-1B) have been identified in chickens (Lynn *et al*., 2004; Van Dijk *et al*., 2005; Xiao *et al*., 2006; Achanta *et al*., 2012). The antimicrobial

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activities of synthesized peptides of several AMPs against bacteria and fungi have been demonstrated (van Dijk *et al*., 2012; Yacooub *et al*., 2015; Lee *et al*., 2016). We reported that the genes of ten *AvBDs* (*AvBD1–8*, *10,* and *12*) were expressed in chick ileum and cecum, and the expression levels of eight *AvBDs* declined during embryo development and chick growth (Terada *et al*., 2018). The expression of four CATHs was also detected in chick proventriculus and cecum (Mohammed *et al*., 2016). Previous studies have shown the presence of AvBD2 and CATH2 in the mucosal leukocytes (van Dijk *et al*., 2012; Cuperus *et al*., 2016; Terada *et al*., 2018), AvBD8 in the luminal epithelium cells and villi (Rengaraj *et al*., 2018), and AvBD9 in the entero-endocrine cells in the luminal and crypt epithelium in the embryo and chick intestine (Cuperus *et al*., 2016). The expression of AvBDs and CATHs in the chick intestine was affected by infection with *Salmonella*, *Campylobacter*, and *Eimeria* in *in vivo* studies (Hong *et al*., 2012; Shao *et al*., 2016; Taha-Abdelaziz *et al*., 2017). IL-1β, a pro-inflammatory cytokine, may play a role in the regulation of AvBDs because expression of AvBDs was upregulated by IL-1β in the ovarian and oviduct tissues (Abdelsalam *et al*., 2012; Sonoda *et al*., 2013). Meanwhile, anti-inflammatory cytokines, such as transforming growth factor (TGF) β may play roles in the maintenance of physiological inflammation in the gut, which is the homeostatic balance between tolerance to microbiota and the reactivity to pathogen invasion (Kogut *et al*., 2018). Thus, it is assumed that the expression of AMPs in the intestinal mucosa is affected by a luminal microorganism complex together with pro-inflammatory and anti-inflammatory cytokines.

In chicken, mice, and piglets, live probiotics have been suggested to induce dynamic changes in the microbiome, and a factor regulating them may be the short-chain fatty acids in the intestinal contents (Wang *et al*., 2019; Vemuri *et al*., 2019; Neijat *et al*., 2019; Cao *et al*., 2019). Additionally, butyrate has been reported to enhance not only epithelial cell proliferation with the development of villi (Guilloteau *et al*., 2010; Ahsan *et al*., 2016) but also the mucosal barrier by tight junctions (Peng *et al*., 2009; Guilloteau *et al*., 2010) and induction of host defense peptide expression including AvBDs and CATHs in the chick intestine (Peng *et al*., 2009; Sunkara *et al*., 2011, 2014). Probiotic bacteria, such as *Lactobacillus* and *Clostridium butyricum*, may play a role in increasing those short-chain fatty acids in the gut. Nii *et al*. (2020) suggested that the administration of *Lactobacillus reuteri* induces the growth of ileum villus and the expression of the tight junction-related molecules in the crop and duodenum of chicks. Zhang *et al*. (2016) reported that an *Escherichia coli* challenge induces a decrease in TNF-α and IL-4 concentration in chick jejunal mucosa, whereas *C. butyricum* suppressed that decrease in TNF-α and IL-4. Thus, the probiotics *L. reuteri* and *C. butyricum* may have benefits that enhance the immunodefense and mucosal barrier system in the chick intestine. However, the specific effects of these two probiotics on the innate immune system in the chick intestine remain unknown. This knowledge is necessary to consider a strategy for the development of probiotic products for chicks.

The aim of this study was to determine the effects of these live probiotics on the expression levels of factors involved in the innate immune system in the chick intestine. We examined the effects of live commercial probiotics of *L. reuteri* and *C. butyricum* on the gene expression levels of TLRs, pro- and anti-inflammatory cytokines, and AMPs in chick ileum and cecum.

Materials and Methods

Treatment of Birds and Tissue Collection

Fertilized eggs (Chunky broiler) obtained from a local hatchery (Fukuda Breeders Co., Okayama, Japan) were incubated in a humidified incubator at 37.5℃. After hatching, 1-day-old male chicks were divided into three groups: *L. reuteri* (LR)*, C. butyricum* (CB), and Control (Con) ($n=7$ in each group). They were maintained in a brooding room with a lighting schedule of 23 h light: 1 h dark for 7 days. They were given a commercial starter diet (NichiwaSangyo Co. Ltd., Kobe, Japan) and water *ad libitum*. Chicks in each group were administered an oral gavage with 500 μ*l* water with or without live probiotic materials daily from day 1 to 6 before used on day 7. The solution (500 μ*l*) given in the LR group contained 2×10^9 CFU of *L. reuteri* (10 mg FINELACT, Asahi Calpis Wellness Co., Ltd., Tokyo, Japan) and that in the CB group contained 1.3×10^7 cells of *C. butyricum* (Ace Bio Product Co., Ltd., Nagano, Japan). Control group chicks were given only deionized water. On day 7, chicks in all groups were euthanized using carbon dioxide, and their ileum and cecum were collected for examination. This study was approved by the Hiroshima University Animal Research Committee (No. C15-16).

RNA Isolation and cDNA Preparation

Total RNA was extracted using Sepasol RNAI Super (Nacalai Tesque, Inc.), as described by the manufacturer's instructions. The extracted total RNA samples were dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and stored at −80℃ until use. The RNA samples were treated with RQ1 RNase-free DNase mixture (Promega Co., Madison, WI, USA; 1 μg total RNA, 1× DNase buffer, and 1 U DNase in 10 μ*l*) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA), programmed at 37℃ for 30 min and then at 65℃ for 10 min with 1 U RQ1 DNase Stop Solution (Promega Co.). The concentration of RNA in each sample was measured using Nano Drop Lite (Thermo Fisher Scientific., Waltham, MA, USA). The RNA samples were reverse-transcribed using Rever-Tra Ace (Toyobo Co., Ltd., Osaka, Japan) as per manufacturer's instructions. The reaction mixture (10 μ*l*) comprised 0.5 μg total RNA, 1× 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 Rever Tra Ace. The reverse transcription was performed at 42℃ for 30 min, followed by heat inactivation at 99℃ for 5 min using a programmable thermal controller. Finally, the

cDNA samples were stored at −20℃ until use.

Real-time PCR

Real-time PCR was performed using the Aria Mix Real-time PCR system (Agilent Technologies Japan. Ltd., Tokyo, Japan). The reaction mixture (10 *μl*) consisted of 1 *μl* cDNA, 1×Brilliant Ⅲ SYBR Green QPCR Mix (Agilent Technologies Japan, Ltd.), 0.25 *μ*M of each primer and water. The primer sequences used in this study are shown in Table 1 (Supplementary data), and two different PCR protocols were used. *TLR2-1*, *4*, *5*, and *21* were selected as the receptors recognizing bacterial molecular patterns. *IL-1β* was examined as a pro-inflammatory cytokine. *AvBD1, 2, 4, 6,* and *7* were selected because their PCR products were detectable. The primers for *CATH2* and *3* were designed against specific sequences of these genes, whereas *CATH1/3* was designed against the sequence shared by both *CATH1* and *CATH3* since the two *CATH*s shared >90% similarity throughout their sequence (Xiao *et al*., 2006). *CATH-B1* was omitted since negligible PCR products were formed. The first protocol was 50 cycles at 95℃ for 5 s, and 58℃ (*TGFβ2* and *4*), 60℃ (*RPS17*, *TGFβ3, CATH1/3*, *2*, *3*, *TLR2-1*, *4*, *5,* and *21*) or 62℃ (*AvBD2*, *4*, *6,* and *7*) for 10 s. The second protocol was 50 cycles at 95℃ for 5 s, and 55℃ (*AvBD1*) and 72℃ for 10 s each. Real-time PCR data were analyzed using the $2^{-\Delta\Delta ct}$ method to calculate the relative level of gene expression in each sample and were expressed as ratios of the *RPS17* housekeeping gene (Livak and Schmittgen, 2001). RNA from the control group was used as a standard sample.

Statistical Analysis

The significance of differences in the real-time PCR data between Con and probiotics treatment (LR and CB) groups was examined using the Kruskal-Wallis test followed by the Steel test (Con was compared with the LR and CB groups). Differences were considered significant when the *P*-value was <0.05.

Results

Figure 1 shows the effects of probiotic treatments on gene expression levels of *TLR2-1*, *4*, *5*, and *21* in the ileum and cecum. In the ileum, the expression levels of *TLR2-1* in the CB group and *TLR5* in the LR and CB groups were significantly higher than that of the Con group (Fig. 1a, e). The expression of the four *TLRs* in the LR and CB groups were not significantly different from that of the Con group in the cecum (Fig. 1b, d, f, h).

Figure 2 shows the effects of probiotic treatments on the gene expression levels of *IL-1β* and *TGFβ2-4* in the ileum and cecum. In the ileum, the expression levels of *IL-1β* in the LR and CB groups and that of *TGFβ2* in the CB group were higher than that in the Con group (Fig. 2a, c). On the contrary, in the cecum, the expression levels of *TGFβ3* in the LR group and that of *TGFβ4* in the LR and CB groups were higher than that in the Con group (Fig. 2f, h).

Figure 3 shows the effects of probiotic treatments on the gene expression levels of *AvBDs* in the ileum and cecum. In the ileum, the expression levels of all the tested *AvBDs* were not significantly different between Con and probiotics treatment (LR and CB) groups (Fig. 3a, c, e, g, and i). On the contrary, in the cecum, the expression levels of *AvBD1* in the CB group and that of *AvBD4* in the LR group were significantly higher than that of the Con group (Fig. 3b and f).

Figure 4 shows the effects of probiotic treatments on the gene expression of *CATHs* in the ileum and cecum. In the ileum, the expression levels of *CATH2* were significantly lower in the LR group than in the Con group (Fig. 4c). In the cecum, the expression levels of *CATH3* were significantly higher in the CB group than that in the Con group (Fig. 4f).

Discussion

Our study reports that the probiotics LR and CB affect the expression levels of TLRs, cytokines, and AMPs in chick ileum and cecum. The following were the major findings: (1) LR significantly increased *TLR5*, *IL-1β*, and *CATH2* in the ileum and *TGFβ3*, *TGFβ4*, and *AvBD4* in the cecum; (2) CB significantly increased *TLR2-1*, *TLR5*, *IL-1β*, and *TGFβ2* in the ileum and *TGFβ4*, *AvBD1*, and *CATH3* in the cecum.

The increase in *TLR2-1* and *TLR5* transcription may result in enhanced ability to recognize gram-positive bacteria, including probiotic bacteria and bacterial flagellin. Wang *et al*. (2013) reported that the live probiotic *Lactobacillus casei* Zhang (LcZ) promotes the transcription of *TLR2*, whereas heat-killed LcZ increases the transcription of *TLR2*, *3*, *4*, and *9* in a murine macrophage cell line. Thus, it is likely that probiotics used in this study modulate the expression of TLRs in the ileum cells as was observed in the mammalian macrophage cell line.

The current results reveal an increase in expression of a pro-inflammatory cytokine (*IL-1β*) in the ileum and also an increase in anti-inflammatory cytokines (*TGFβ2-4*) in the ileum and cecum, by LR and CB treatments. We assume that the balanced expression of pro- and anti-inflammatory cytokines were thus maintained in the chicks treated with LR and CB. This would be necessary for regulation of physiological inflammation that is responsible for normal immune functioning of the intestinal mucosa (Crhanova *et al*., 2011) and maintaining homeostatic balance between tolerance of the microbiota and reactivity to pathogen invasion (Kogut *et al*., 2018).

Treatment with CB promoted the transcription of *AvBD1* and *CATH3*, and LR treatment enhanced transcription of *AvBD4* in the cecum. The pattern molecules in the cell wall of LR and CB may be commonly recognized by *TLR2* because both are gram-positive bacteria. However, treatment with LR and CB affected the transcription of different *AvBD*s and *CATH*s in the cecum and ileum. Thus, the effects of CB and LR on AMP transcription may be regulated not only through TLRs but also by other factors. Short-chain organic acids produced by them could be one such factor. Organic acids produced by probiotic bacteria are known to lower the luminal pH and inhibit the growth of some pathogens, such as *E. coli* (Ohland and MacNaughton, 2010). We assume that changes in the luminal microbiome composition may affect the expression of AMPs in the intestine. Furthermore, it was reported that dietary supplementation with butyrate

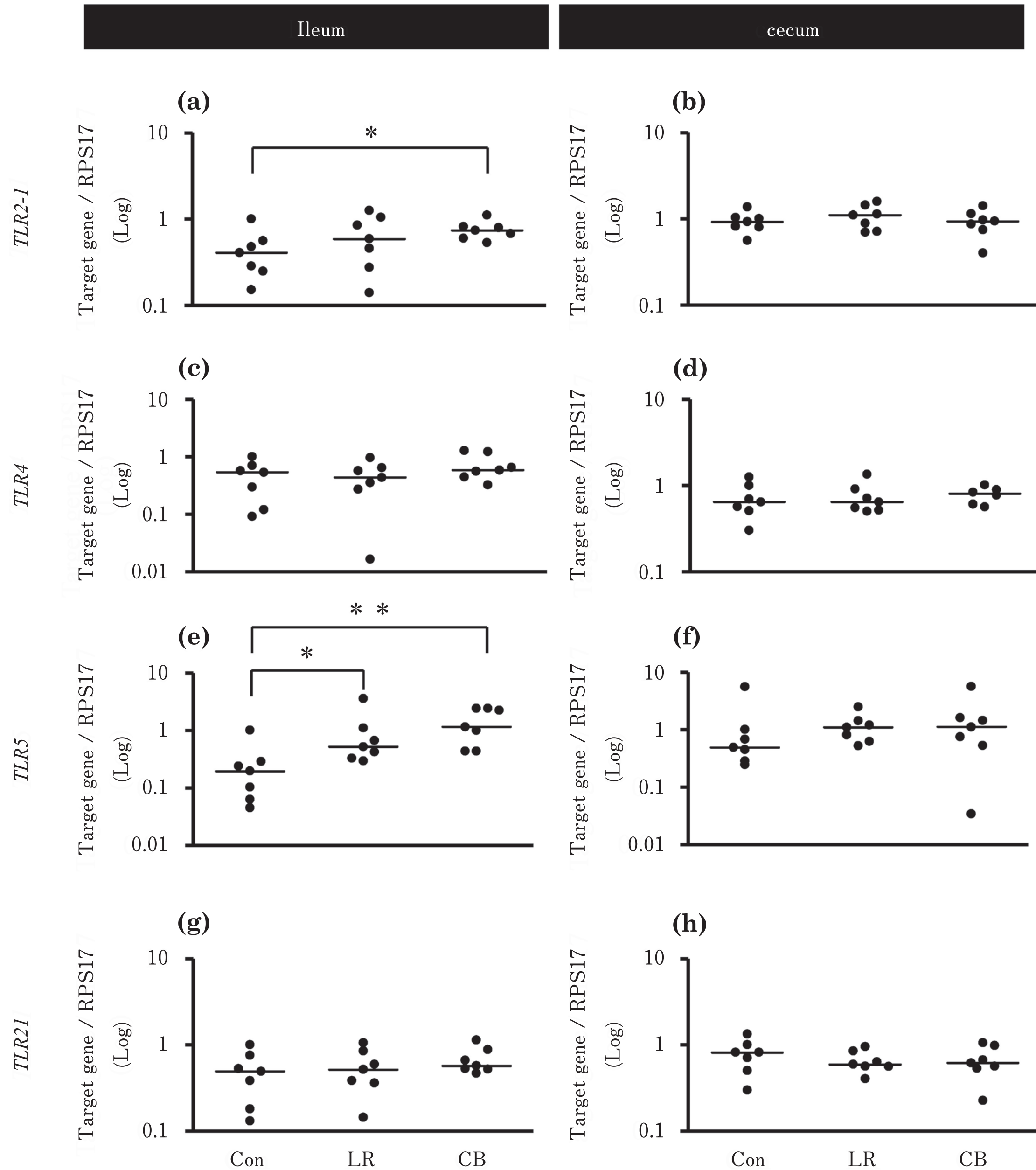

Fig. 1. **Effects of probiotic treatments on the expression levels of *TLRs* in the chick ileum and cecum.** The values are fold changes in gene expression. Chicks in Con, LR, and CB groups were orally administrated with 500 μl deionized water, 2×10^9 CFU *L. reuteri*, and 1.3×10^7 cells *C. butyricum*, respectively [$n=7$ in each group, except for TLR4 data for the cecum in the CB group ($n=6$). The solid bar represents the median value within each group. Asterisks indicate significant differences between the Con group and each probiotic group, determined by Kruskal-Wallis and Steel tests (* $P<0.05$, ** $P<0.01$).

induced *AvBD9*, *AvBD14*, and *CATHB1* in the jejunum and cecum and reduced the colonization of bacteria following experimental infection with *S. enteritidis* in chickens. Butyrate also has synergic effects on the induction of *AvBD9* by cyclic AMP in chick jejunum explants and macrophages. In humans, it is also reported that butyrate upregulates the expression of cathelicidins LL37 in the luminal epithelium of colonocytes (Schauber *et al*., 2003). Thus, we suggest that LR and CB modulate the expression of AMPs in the cecum, probably not only through TLR stimulation but also through

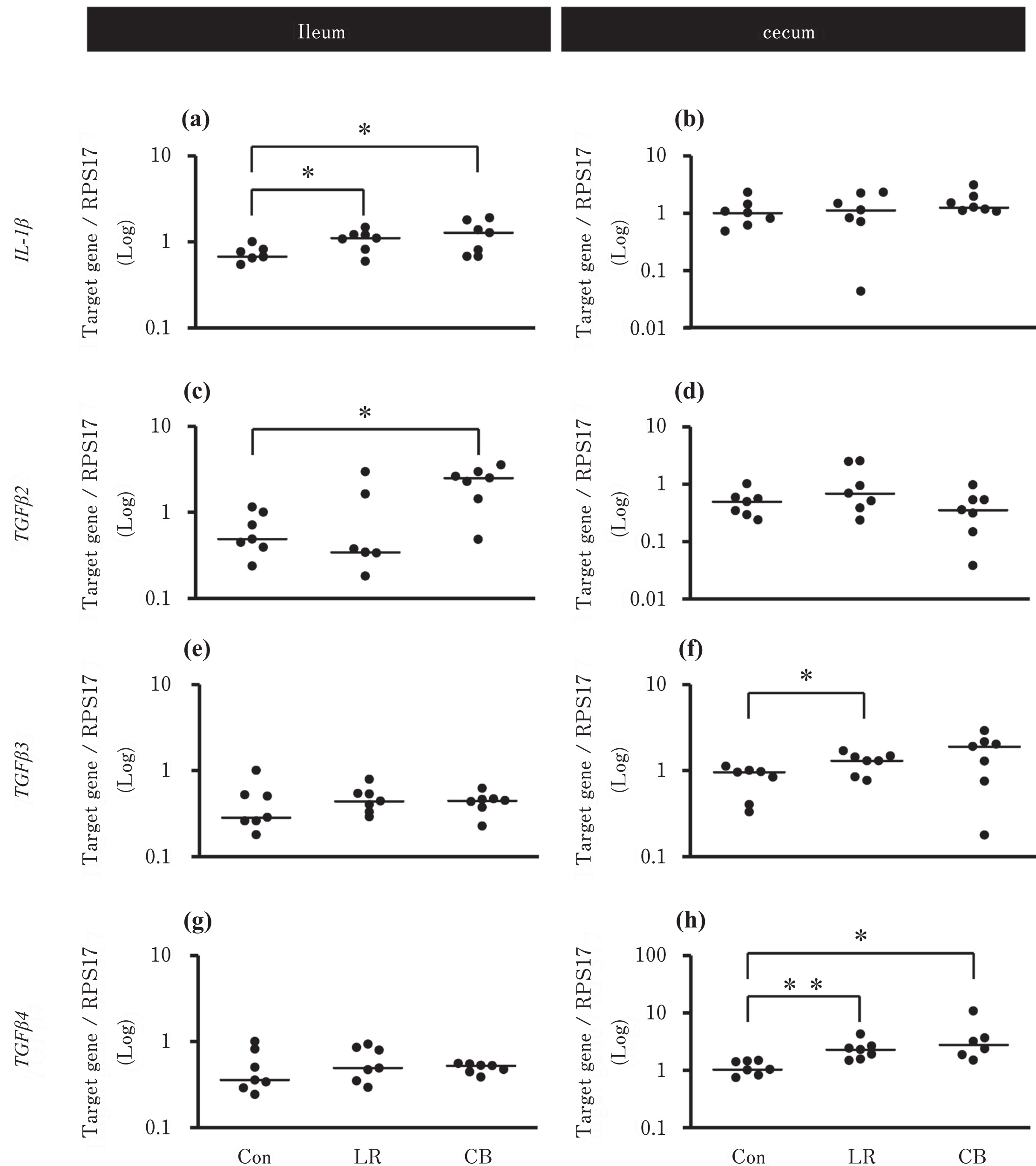

Fig. 2. **Effects of probiotic treatments on the expression levels of pro- and anti-inflammatory cytokines in the chick ileum and cecum.** Chicks in Con, LR, and CB groups were orally administrated with 500 μl deionized water, 2×10^9 CFU *L. reuteri*, and 1.3×10^7 cells *C. butyricum*, respectively ($n=7$ in each group). Asterisks indicate significant differences between the Con group and each probiotic group, determined by Kruskal-Wallis and Steel tests (* $P<0.05$, ** $P<0.01$). See Fig. 1 for other explanations.

organic acids produced by them, whereas the effects on AMP expression may be different between LR and CB. Also, in the current study, there were variations in the expression levels of some genes within LR and CB treatment groups; this may be due to the differences in the response of the expression of the immune factors to probiotics among the chicks.

The modulatory effects of LR and CB supplementation on *AvBD*s and *CATH*s were found only in the cecum, and probable suppressive effect on *CATH2* by LR was observed in the ileum in this study. We reported that supplementation with probiotics in feed (*Streptococcus faecalis*, *Clostridium*

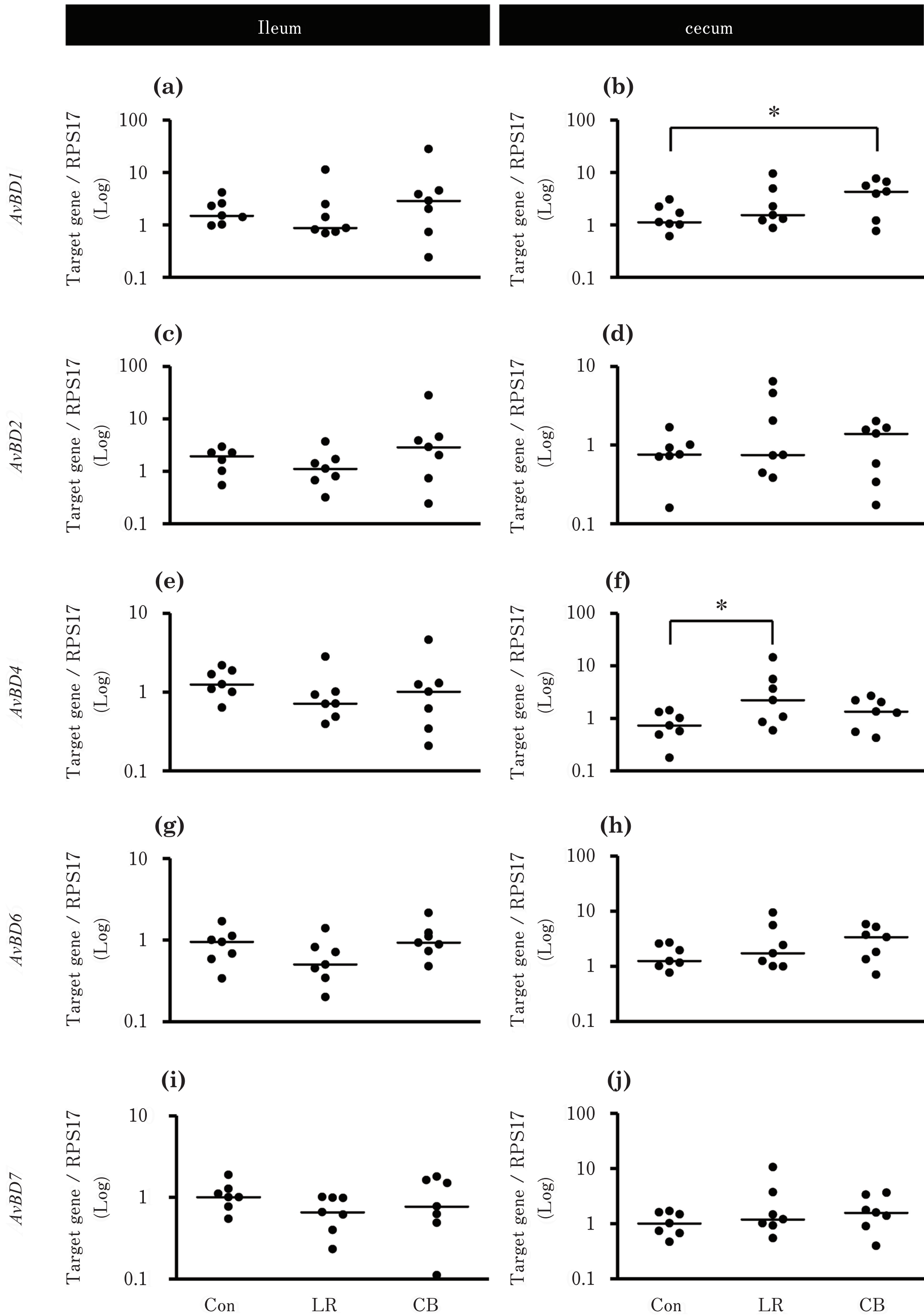

Fig. 3. **Effects of probiotic treatments on the expression levels of *AvBDs* in the chick ileum and cecum.** Chicks in Con, LR, and CB groups were orally administrated with 500 μl deionized water, 2×10^9 CFU *L. reuteri*, and 1.3×10^7 cells *C. butyricum*, respectively [n=7 for each group, except for AvBD2 data in the ileum in the Con group (n=6)]. Asterisks indicate significant differences between the Con group and each probiotic group, determined by Kruskal-Wallis and Steel tests (* $P<0.05$). See Fig. 1 for other explanations.

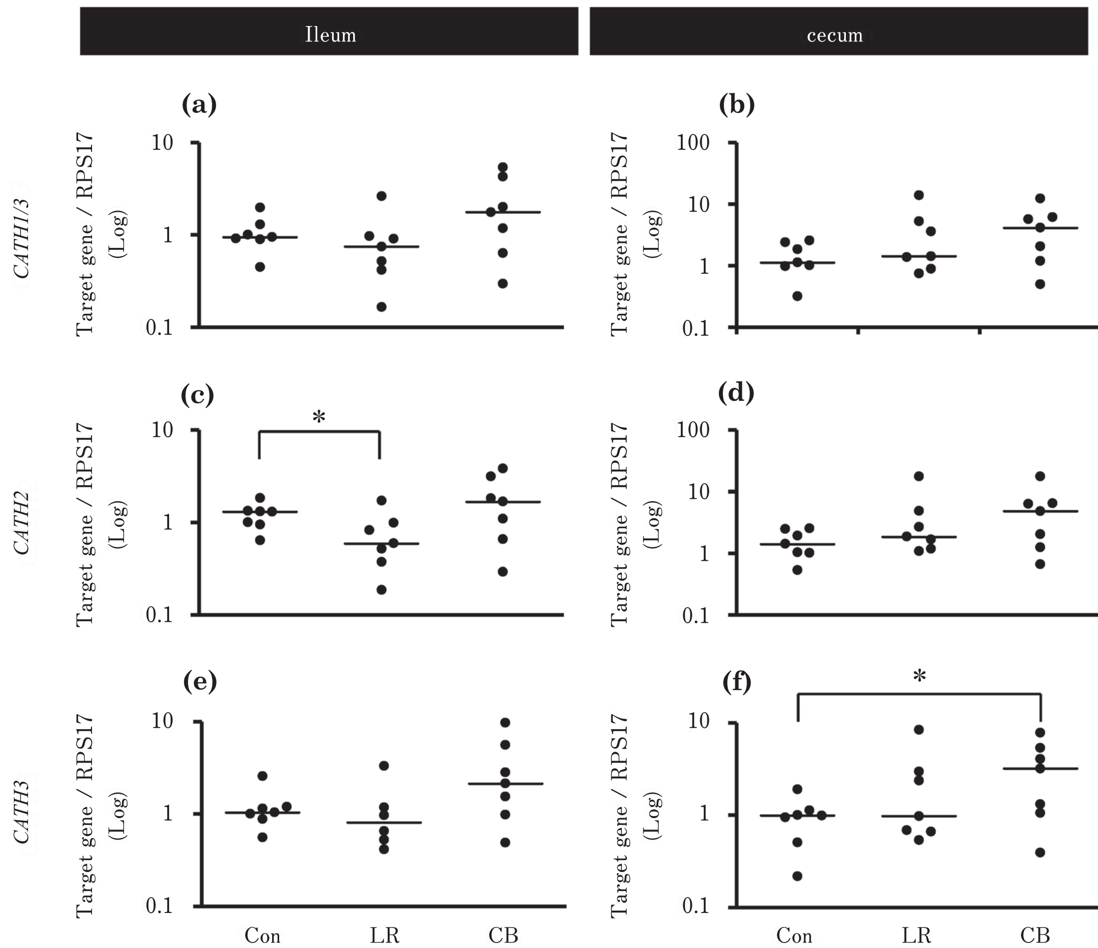

Fig. 4. **Effects of probiotic treatments on the expression levels of *CATHs* in the chick ileum and cecum.** Chicks in Con, LR, and CB groups were orally administrated with 500 μl deionized water, 2×10^9 CFU *L. reuteri*, and 1.3×10^7 cells *C. butyricum*, respectively ($n=7$ in each group). Asterisks indicate significant differences between the Con group and each probiotic group, determined by Kruskal-Wallis and Steel tests (* $P<0.05$). See Fig. 1 for other explanations.

butyricum, and *Bacillus mesentericus*) did not affect the *AvBD* and *CATH* expression in the chick proventriculus (Mohammed *et al*., 2016), whereas expression of *CATH2* in response to LPS was enhanced in the cecum by probiotic feeding (Mohammed *et al*., 2016). Treatment of chicks with probiotics (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Enterococcus feacalis*) suppressed the increase in *AvBD* and *CATH* expression induced by *Salmonella typhimurium* challenge in the cecal tonsil (Akbari *et al*., 2008). Thus, all our findings suggest that the cecum may be more sensitive in AMP expression to probiotic bacteria than upper segments of the gut. We speculate that the higher sensitivities in the cecum associate with the rich microbiota stock in that segment.

In conclusion, we suggest that probiotic LR and CB treatments affect the expression of innate immune molecules (TLRs, pro- and anti-inflammatory cytokines, and AMPs). The significant increase in the expression of *TLR*s in the ileum and AMPs, including *AvBD*s and *CATH*s, in the cecum by these probiotics, may enhance a part of the innate immunodefense system in these tissues of chicks.

Conflicts of Interest

The authors declare no conflict of interest.

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