JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0160064


# Effects of Probiotics on the Expression of Cathelicidins in Response to Stimulation by *Salmonella Minnesota* Lipopolysaccharides in the Proventriculus and Cecum of Broiler Chicks

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The aim of this study was to determine whether probiotic-feeding affected the expression of cathelicidins (*CATHs*), a major family of antimicrobial peptides, in response to lipopolysaccharides (LPS) challenge in the proventriculus and cecum of broiler chicks. One-day-old male Chunky broiler chicks were fed with or without 0.4% probiotics for 7 days (P-group and non-P-group, respectively). Then, they were orally challenged with no LPS (0-LPS), 1 $\mu$g LPS (1-LPS), or 100 $\mu$g LPS (100-LPS) ($n$=5 in all groups) in Experiment 1, and with no LPS or 1 $\mu$g LPS ($n$=6 in all groups) in Experiment 2. Five hours after LPS challenge, the proventriculi and ceca were collected to analyze *CATHs* expression. Expression of *CATHs* was examined at first by reverse transcription-polymerase chain reaction (RT-PCR) using the 0-LPS chicks of non-P-group. The differences in *CATHs* expression upon probiotics-feeding and LPS were analyzed by real time-PCR. All four *CATHs* (*CATH1, 2, 3* and *4*) were expressed in the proventriculus and cecum of chicks. In the proventriculus, the expression of *CATHs* after LPS challenge did not show significant differences between non-P and P-groups in Experiment 1 and 2. In the cecum, the interactions of the effects of probiotics and LPS on the expression of *CATH2* in Experiment 1 and *CATH1* and *2* in Experiment 2 were significant, and their expression in 1-LPS chicks was higher in P-group than in non-P-group. However, *CATH3* and *4* did not show any significant differences between non-P- and P-groups challenged with LPS. These results suggest that probiotics-feeding may stimulate the immunodefense system mediated by *CATH2* and possibly *CATH1* against infection by Gram-negative bacteria in the cecum.

**Key words**: cathelicidins, gut mucosal immunity, probiotics, lipopolysaccharides

*J. Poult. Sci., 53: 298−304, 2016*

## Introduction

The mucosal surface of the gastrointestinal tract after hatching is constantly in contact with various microorganisms (Walker *et al*., 2006). The gut-associated lymphoid tissues have not been fully developed during the first week of life in newly hatched chicks (Miyazaki *et al*., 2007). The immune protection could be provided during the first week of life through maternal antibodies (Kaspers *et al*., 1991) and innate defense system including the synthesis of antimicrobial peptides (AMPs).

AMPs are main parts of the innate immune response to microbial infection. In chickens, two major families of AMPs are defensins and cathelicidins (CATHs) (Lynn *et al*., 2004). AMPs generally consist of less than 100 amino acid residues, mostly cationic and amphipathic in nature, which allows them to bind and disrupt negatively charged microbial membranes leading to death of microbes. Therefore, AMPs are being strongly recommended for the control and prevention of infectious diseases, particularly against antibiotic-resistant bacteria (Hancock and Sahl, 2006). Up to date 4 *CATHs* have been reported in chickens namely, *CATH1* (Lynn *et al*., 2004), *CATH*2 (Van Dijk *et al*., 2005), *CATH*3 (Xiao *et al*., 2006a) and *CATH*4 (Goitsuka *et al*., 2007; Achanta *et al*., 2012). All four chicken *CATHs* are believed to be capable of killing a broad range of bacteria including antibiotic-resistant strains (Xiao *et al*., 2006a, b; Van Dijk *et al*., 2009; Rodríguez-Lecompte *et al*., 2012).

Innate immune responses are stimulated by pathogen-associated molecular pathogens (PAMPs) via Toll-like receptors (TLRs) (Werling and Coffey, 2007). Chicken TLRs are important in the recognition of PAMPs to induce the production of pro-inflammatory cytokines and antimicrobial

Received: April 16, 2016, Accepted: May 30, 2016
Released Online Advance Publication: June 25, 2016
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peptides and to upregulate the expression of co-stimulatory molecules that may initiate adaptive immunity responses (Werling and Jungi, 2003; Yoshimura, 2015). Among these TLRs, TLR4 recognizes lipopolysaccharides (LPS) from Gram-negative bacteria, whereas it requires CD14 to accept the complex of LPS and LPS binding protein (Nerren *et al*., 2010; de Zoete *et al*., 2011).

Manipulation of the gut microbiota of chickens by administration of probiotic bacteria may help to control enteric bacterial infections, including those caused by *Salmonella enterica serovar Typhimurium* (Mead, 2000). In chickens, feeding with probiotic species such as *Lactobacillus*, *Streptococcus*, and *Clostridium* may have beneficial effects on broiler performance (Ashayerizadeh *et al*., 2009) as well as on the modulation of intestinal microflora and their genes (microbiome) to reduce pathogens (Mountzouris *et al*., 2007; Higgins *et al*., 2011; Oakley *et al*., 2014). Probiotics have also been reported to modulate the expression and localization of avian $\beta$-defensins (AvBDs) in the mucosal tissue of the gastrointestinal tract (Akbari *et al*., 2008; Mohammed *et al*., 2015). We expected that probiotics-feeding may also enhance the functions to express *CATHs* in the gut mucosa of chicks.

However, it remains to be established whether probiotics-feeding affects the ability of the gut mucosa to express *CATHs* in chicks. The aim of this study was to determine whether the feeding of probiotics affects the expression of *CATH*s in response to LPS challenge in the proventriculus and cecum of broiler chicks.

## Materials and Methods

### *Treatments of Birds and Tissue Collection*

*Experiment 1:* One-day-old male broiler chicks (Chunky broilers) were purchased from a local hatchery (Fukuda Poultry, Okayama, Japan). They were divided into 2 groups, fed with or without 0.4% probiotics, namely probiotic group (P-group) and non-probiotic group (non-P-group). Chicks in the non-P-group were given a commercial starter diet (Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) containing 0.4% (wt/wt) corn starch, whereas chicks in the P-group were given the starter rations containing 0.4% (wt/wt) probiotics (Toaraze for chickens; Toa Pharmaceutical Co. Ltd., Tokyo, Japan). The Toaraze for chickens contained *Streptococcus faecalis* ($>1\times10^8$/g), *Clostridium buthricum* ($>1\times10^7$/g), and *Bacillus mesentericus* ($>1\times10^7$/g). Chicks were maintained in a brooding room under a lighting condition of 23 h light/1 h dark for 7 days. The chicks were reared with feeds with (P-group) or without (non-P-group) probiotics and water *ad libitum*. On day 7, the chicks in each group were divided into 3 subgroups, namely 0-LPS, 1-LPS, and 100-LPS groups, which were given 1 mL of clean water containing 0, 1, or 100 $\mu$g LPS through oral gavage, respectively. Five hours after LPS challenge, the chicks were euthanized using carbon dioxide, and the proventriculi and ceca were collected ($n=5$ in all groups).

*Experiment 2:* This experiment was carried out to confirm the results of Experiment 1 for 0-LPS and 1-LPS using the previous design of Experiment 1. The chicks were fed feeds with (P-group) or without (non-P group) probiotics and water *ad libitum* for 7 days. Chicks were divided into 2 subgroups, namely 0-LPS and 1-LPS groups, which were given 1 mL of clean water containing 0 $\mu$g LPS or 1 $\mu$g LPS, respectively ($n=6$ in all groups).

The LPS used in this study originated from *Salmonella Minnesota R595* (Re-mutant; by ultracentrifugation; Wako pure chemical industries Ltd. Osaka. Japan). Chicks were handled in accordance with the regulations of Hiroshima University Animal Research Committee.

### *RNA Isolation and cDNA Preparation*

The mucosal tissues of proventriculi and ceca were collected immediately after birds were euthanized. The proventriculus was opened longitudinally by a scissors and washed by a cold and autoclaved PBS, then spread on a sterilized glass slide. The thick mucosal layer of the proventriculus was carefully cut with scissors and collected. The cecum was longitudinally opened with scissors and was also washed with PBS, and then spread on a sterilized glass slide. The mucosal layer of the cecum was carefully and gently scrubbed by a sharp blade and collected.

The collected mucosal tissues of the proventriculus and cecum were used for total RNA extraction by Sepazol RNA I super according to the manufacturer's directions (Nacalai Tesque Inc., 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℃ 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) in a 10 $\mu$L reaction mixture (1 $\mu$g of total RNA, 1× DNase buffer, and 1U DNase) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA), programmed at 37℃ for 45 min and 65℃ for 10 min. The concentration of RNA in each sample was measured once more after DNase treatment using Gene Quant Pro (Amersham Pharmacia Biotech). The RNA samples were then reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions to obtain cDNA. The reaction mixture (10 $\mu l$) consisted of 1 $\mu$g of total RNA, 1× Reverse Transcription buffer, 1 $\mu$M deoxyribonucleotide triphosphate (dNTP) mixture, 20 U RNase inhibitor, 0.5 $\mu$g of oligo (dt) 20 and 50 U Rever Tra Ace. The reverse transcription was performed at 42℃ for 30 min, followed by heat inactivation for 5 min at 99℃ using a programmable thermal controller (PTC-100; MJ Research).

### *Identification of CATHs Expression*

The reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to determine the expression of *CATHs* in the proventriculus and cecum using the cDNA of the chicks from control group of Experiment 1 (0-LPS of non-P-group; $n=5$). The reaction mixture (25 $\mu$L) containing 0.5 $\mu$L of cDNA, 1× PCR buffer, 0.2 $\mu$M dNTP mixture, 0.5 $\mu$M of each primer (forward and reverse), and 0.125 U Takara Taq (Takara Bio. Inc., Shiga, Japan) was prepared.

Table 1. **Primers used for PCR analysis of *CATHs* and their accession numbers**

| Gene | Sequence of forward and reverse primers | Accession no. | Expected product size (bp) |
|---|---|---|---|
| *CATH1* | F: GCTGTGGACTCCTACAACCAAC<br>R: GGAGTCCACGCAGGTGACATC | NM_001001605.3 | 261 |
| *CATH2* | F: CAAGGAGAATGGGGTCATCAG<br>R: CGTGGCCCCATTTATTCATTCA | NM_001024830.2 | 221 |
| *CATH3* | F: GCTGTGGACTCCTACAACCAAC<br>R: TGGCTTTGTAGAGGTTGATGC | NM_001311177.1 | 352 |
| *CATH4* | F: CCGTGTCCATAGAGCAGCAG<br>R: AGTGCTGGTGACGTTCAGATG | NM_001271172.1 | 170 |
| *RPS17* | F: AAGCTGCAGGAGGAGGAGAGG<br>R: GGTTGGACAGGCTGCCGAAGT | NM_204217 | 136 |

*CATHs* primers used for PCR are presented in Table 1. The PCR cycle parameters were 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for all *CATHs* for 45 s, and extension at 72℃ for 45 s, followed by a final extension at 72℃ for 7 min. The PCR products were separated by electrophoresis on 2% (wt/vol) agarose gels containing 0.025% (wt/vol) ethidium bromide and photographed under UV illumination.

***Analysis of Differences in CATHs Expression Among Treatments***

The expression level of the *CATHs* detected by RT-PCR analysis in both the proventriculus and cecum specimens was further analyzed by real-time PCR using the Roche Light Cycler Nano system (Roche Applied Science, Indianapolis, IN, USA). The reaction mixture (20 $\mu$L) containing 1 $\mu$L of cDNA, 10 $\mu$L of Thunder Bird SYBR qPCR Mix (Toyobo Co. Ltd., Osaka, Japan), 1 $\mu$M of each primer, and Milli Q water were mixed into PCR tubes (Roche Diagnostics GmbH, Mannheim, Germany). The thermal protocols for PCR were 50 cycles at 95℃ for 10 s; and 60℃ (*CATHs* and *RPS17*), for 30 s. Real-time PCR data were analyzed using the $2^{-\Delta\Delta ct}$ method to calculate the relative level of *CATHs* expression in each sample and were expressed as ratios in relation to the *RPS17* housekeeping gene (Livak and Schmittgen, 2001). An RNA sample of control chicks (0-LPS) from the non-P-group was used as a standard sample.

***Statistical Analysis***

The significance of interaction between probiotics-feeding and LPS treatments was examined by two-way ANOVA. Then, when the interaction was significant, the difference between non-P-group and P-group at each different LPS treatment was examined by t-test. Differences were considered significant when the P value was $<0.05$.

## Results

***Identification of CATHs Expression in the Proventriculus and Cecum of Chicks***

The expression profile of *CATHs* in the mucosal tissue of the proventriculus and cecum revealed that *CATHs* (*CATH1, 2, 3* and *4*) were expressed in the proventriculus and cecum of broiler chicks (Fig. 1a and b).

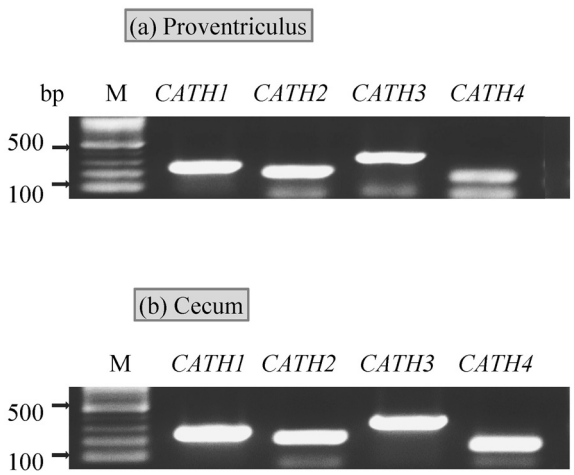


Fig. 1. **Reverse transcription-PCR analysis of *CATHs* expression in the proventriculus (a) and cecum (b) of chicks.** Total RNA samples were collected from the mucosal tissue of the proventriculus and cecum of 0-LPS chicks of non-P-groups.

***Analysis of Differences in CATHs Expression Among Treatments***

In Experiment 1, the effects of three different doses of LPS challenge on the expression of *CATHs* in the proventriculus and cecum of P- and non-P-groups were examined (Fig. 2). In the proventriculus, the interaction between probiotics and LPS treatments for the induction of *CATH1-4* was not significant among non-P and P-groups treated with 0- , 1- or 100-LPS (Fig. 2a, c, e and g). In the cecum, the interaction of probiotics and LPS treatments for the induction of the expression of *CATH2* was significant ($P=0.0053$) (Fig. 2d), but not for the expression of the other *CATHs* (Fig. 2b, f and h). In addition, *CATH2* expression in the 1-LPS chicks was significantly higher in P-group than non-P-group (Fig. 2d).

In Experiment 2, the interaction of probiotics and LPS treatments for the induction of the 4 *CATHs* expressions was not significant in the proventriculus (Fig. 3a, c, e and g).

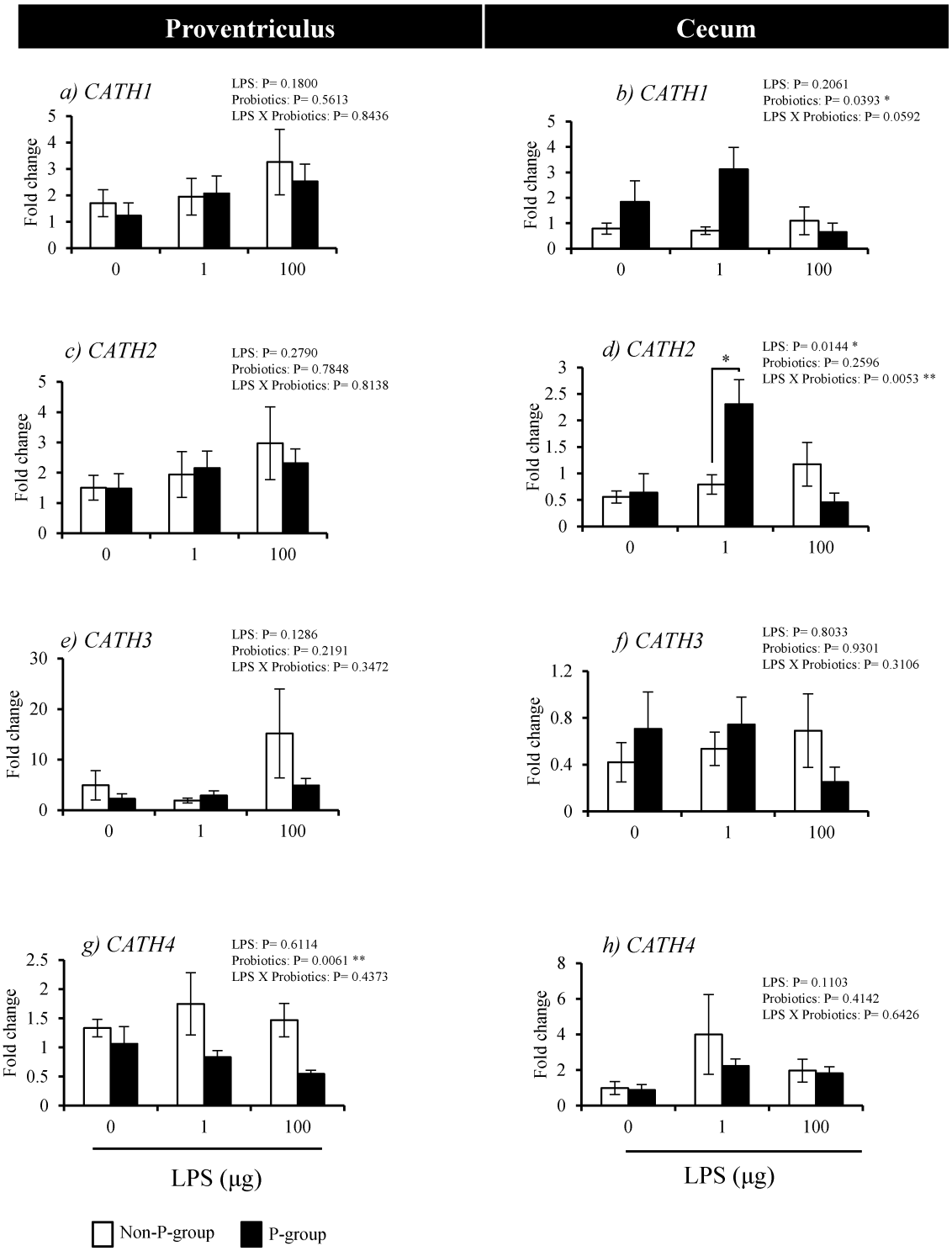


Fig. 2. **Effects of probiotics-feeding on *CATHs* expression of in response to LPS in the proventriculus and cecum of chicks (Experiment 1).** Values are mean±S.E. of fold changes in expression ($n$= 5). Non-P and P-groups were fed 0% and 0.4% probiotics, respectively and challenged with 0, 1 or 100$\mu$g LPS (0-, 1-, and 100-LPS groups). * Values are significantly different between P-group and non-P-group ($P<0.05$).

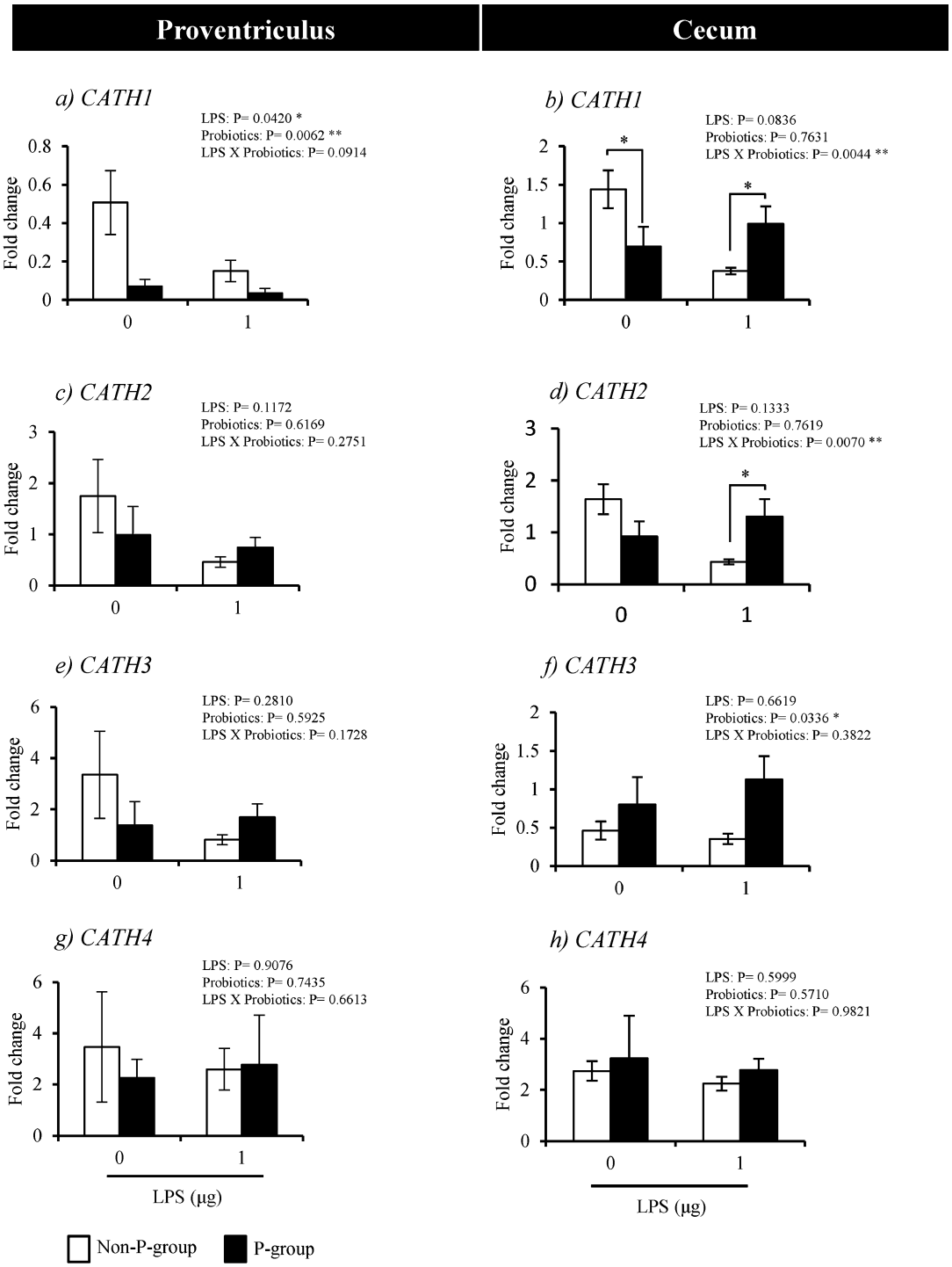


Fig. 3. **Effects of probiotics-feeding on the mRNA expression of *CATHs* in response to LPS in the proventriculus (a, c, e and g) and cecum (b, d, f and h) of chicks (Experiment 2).** Values are mean± S.E. of fold changes in expression ($n$=6). Non-P and P-groups were fed 0% and 0.4% probiotics, respectively and challenged with 0 or 1 $\mu$g LPS (0- and 1-LPS groups). * Values are significantly different between P-group and non-P-group ($P$<0.05).

However, in the cecum, there were significant interactions between LPS and probiotics treatments for the induction of the expressions of *CATH1* ($P=0.0044$) and *CATH2* ($P=0.0070$) (Fig. 3b and d). The expression of *CATH1* in the 0-LPS chicks was lower in P-group than in non-P-group chicks (Fig. 3b), whereas, its level in the 1-LPS chicks was significantly higher in P-group than in non-P-group (Fig. 3b). The expression level of *CATH2* in 1-LPS chicks was higher in P-group than in non-P-group (Fig. 3d). The interactions between LPS and probiotics treatments for the induction of the expression of *CATH 3* and *4* were not significant (Fig. 3f and h).

## Discussion

In this study we report the effects of probiotics-feeding on the response of the proventricular and cecal mucosa to LPS in term of *CATHs* expression. The major findings of this study are: (1) four *CATHs* expression was detectable in the mucosal tissue of the proventriculus and cecum of broiler chicks; (2) *CATH2* in the cecum showed a higher expression level in response to 1μg LPS challenge in the P-group than in the non-P-group. These results showing the expression of *CATHs* in the proventriculus and cecum supports the previous studies that reported *CATHs* are expressed in the mucosal tissues of the digestive and respiratory of chickens (Van Dijk *et al*., 2005; Xiao *et al*., 2006a; Goitsuka *et al*., 2007). The synthesized *CATHs* in the digestive tract may play roles in defense against pathogens since they have a broad spectrum of antimicrobial activities (Van Dijk *et al*., 2005; Xiao *et al*., 2006a, b). The expression levels of any *CATHs* with no LPS challenge was not higher in the P-group than in non-P-group in both the proventriculus and cecum, and *CATH1* expression in the cecum was lower in the P-group than in non-P-group in Experiment 2. These results suggest that probiotics themselves did not upregulate *CATHs* expression as reported for the *AvBDs* expression in the proventriculus (Mohammed *et al*., 2015).

The current study revealed that in the cecum there were significant interactions between probiotics-feeding and LPS treatments in the induction of *CATH2*, and its expression level in response to 1 $\mu$g LPS was higher in the P-group than in non-P-group, commonly in Experiments 1 and 2. The significant interaction and higher expression level in the P-group than in non-P-group were identified also for *CATH1* in the Experiment 2. These results suggest that probiotics may enhance the ability to respond to LPS for the induction of *CATH2*, and also possibly that of *CATH1*, in the cecum. We hypothesize that the cellular functions for recognizing LPS and expressing *CATHs* in the cecum may be modulated by probiotics-feeding in that process.

The challenge with a greater dose of LPS (100 $\mu$g) did not show differences in *CATH2* expression in the cecum between P-group and non-P-group in Experiment 1. Although the reason for these results is not known, the function to recognize LPS or to express *CATH2* may be reduced when the tissues were challenged with a greater dose of LPS even in the chicks fed with probiotics. In the vaginal cells, AvBDs expression was upregulated by LPS, but the response was decreased by a higher dose of LPS treatment (Sonoda *et al*., 2013).

In contrast to cecum, *CATHs* expression in the proventriculus was not significantly affected by probiotics-feeding and LPS challenge. The effects of probiotics may be weak in the proventriculus since the probiotics bacteria may not be able to adapt and proliferate in the acidic medium of the proventriculus. We have reported that probiotics-feeding did not affect the expression of *AvBD12*, wherease the AvBD12 protein density in the surface epithelial cells was lowered by probiotics-feeding, suggesting that they were secreted more in chicks fed with probiotics (Mohammed *et al*., 2015). Although the exact reason why LPS did not affect the *CATHs* expression in the proventriculus in both P- and non-P-groups is not known, we assume that mucous substances on the mucosal surface protected the tissue from binding of LPS.

In conclusion, the current study showed that probiotics-feeding alone did not affect the expression of *CATHs* in the mucosa of the proventriculus and cecum. However, the response of *CATH2* expression, and possibly also *CATH1* expression, to LPS challenge may be enhanced by probiotics-feeding in the cecum. Thus, probiotics may enhance the immunodefense system mediated by *CATHs* against infection by Gram-negative bacteria in the cecum of broiler chicks.

## Acknowledgments

The authors thank Toa Pharmaceutical Co., Ltd. (Tokyo, Japan) for kindly giving us probiotics (Toaraze for chickens). This work was supported by a Grant-in-Aid for Challenging Exploratory Research from the Japan Society for the Promotion of Science (No. 25660213).

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