

Doctoral Thesis

**Studies on probiotics effects on innate
immune functions in the gastrointestinal
tract of broiler chicks**

(SUMMARY)

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In chickens, the gastrointestinal tract is a vital system which responsible for digestion and nutrient uptake as well as immune functions. A well-functioning and healthy gut is necessary for the optimum performances of the birds. The gut mucosal tissue plays an important role in providing an effective barrier between the luminal contents and the host internal tissues by means of epithelial cells tight junctions, mucous layer secretion, antimicrobial peptides secretion and lymphocytic activity. To support this intestinal mucosal barrier functions it is essential to keep the dynamic balance between the intestinal epithelial cells, microbiota and immune cells in the intestine. The use of antibiotics has been widely spread in poultry industry for decades to maintain the balance of the gut and to improve the growth performance of chicken. However, due to the appearance of antibiotics resistant bacteria in chickens in addition to human health complication issues, the use of antibiotics was banned. Vaccination also is another method for controlling infections but is effective only against specific pathogens. Therefore, many alternatives are under investigation for their possible beneficial effects in poultry industry to maintain the gut health and enhance the immune defense functions to promote the performance of birds including probiotics. The aim of this study was to determine the effects of probiotics-feeding on the mucosal barrier functions including tight junction proteins, antimicrobial peptides and cytokines expression.

1. Expression of claudins (tight junction proteins) in the digestive tract of broiler chicks and effects of probiotics on their expression

Intestinal epithelial integrity is vital for nutrition absorption and host defense against pathogens. Mucosal barrier systems formed by epithelial cell junctions, mucin layer, and leukocyte activity, have primary roles to prevent infection. The aim of this study was to examine the expression of tight junction proteins namely claudins (*CLD1* and *5*), and the effects of probiotics-feeding on their gene expression in the gastrointestinal tract of broiler chicks. To investigate the two main items of this study, two experiments were designed. In Experiment 1, the expression of *CLD1* and *2* was examined in 7-days-old male broiler chicks. Chicks were fed *ad libitum* with starter rations. At day 7 (D7) the proventriculus and intestine were collected to examine the existence of *CLD1* and *5* gene expression. In Experiment 2, the 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 consisting of *Streptococcus faecalis*, *Clostridium buthricum* and *Bacillus mesentericus*, respectively, from day 0 (D0; at one-day-old) to D14. The proventriculus and intestine were collected in all groups at D0, D7 and D14 for analysis of *CLD1* and *5* expressions in response to feeding with probiotics. The expression of *CLDs* genes was examined by RT-PCR and changes in the expression upon probiotics-feeding were examined by real-time PCR. In Experiment 1, in the proventriculus,

ileum, cecum and colon the expression of *CLDI* and 5 was detectable. In Experiment 2, the expression of the *CLDI* and 5 did not show significant differences between control and probiotics groups at D7 and D14 in each intestinal segments except in the colon at D7, the expression of *CLDI* in the colon was higher in probiotic group I than control and probiotics group II. The expression of *CLD5* was higher in probiotic group I than probiotics group II. These results suggest that, although probiotics-feeding may not affect the gene expression of *CLDI* and 5 in the proventriculus, ileum and cecum, it affected the expression of *CLDI* and 5 in the colon and play role in modulating the barrier defense mechanisms of mucosal epithelium carried out by the tight junctions in chicks.

2. Expression of avian β -defensins and effects of probiotics on their expression in the digestive tract of broiler chicks with special reference to localization of AvBD12 in the chick proventriculus

Avian β -defensins (AvBDs), a member of antimicrobial peptides family, that play important role in the innate immunodefense system in the gut of chicks. Probiotics may affect the immune functions in the gut mucosa to suppress infections. The innate immunodefense functions that are carried out by antimicrobial peptides in chicks has unique features. The aim of this study was (1) to examine the expression profile of *AvBDs* in the gastrointestinal tract of broiler, (2) to determine the effects of probiotics-feeding on the gene expression of *AvBDs* in the proventriculus and cecum and (3) localization of ir-AvBD12 in the proventriculus of broiler chicks. In Experiment 1, male broiler chicks were reared from D0 to D7 post-hatching and chicks were fed only with starter ration. At D7, the proventriculus, ileum, cecum and colon were collected for *AvBDs* expression analysis (n= 10). In Experiment 2, 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 to D14. The proventriculus and cecum in all groups were collected at D0, D7 and D14 for analysis of the effects of probiotics on *AvBDs* expression. In Experiment 3, the proventriculi were collected from the same birds of Experiment 2 for AvBD12 protein localization. The expression of *AvBDs* genes in the gastrointestinal tract was examined by RT-PCR and changes in the expression upon probiotics-feeding were examined by real-time PCR. The AvBD12 localization was examined by immunohistochemistry. Out of 14 *AvBDs* genes, seven *AvBDs* in the proventriculus and colon (*AvBD1*, 2, 4, 6, 7, 10 and 12), nine *AvBDs* (*AvBD1*, 2, 3, 4, 5, 6, 7, 10 and 12) in the ileum and eight *AvBDs* (*AvBD1*, 2, 4, 5, 6, 7, 10 and 12) in the cecum were identified. The expression level of the detected genes did not show any significant differences between control and probiotics groups at D7 and D14 in the proventriculus and cecum. The ir-AvBD12 was localized in the surface epithelium and cells in the connective tissues of proventricular glands. The ir-AvBD12 density in the 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 the 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 did not affect the gene expression of *AvBDs* in the proventriculus and cecum, it may affect *AvBD12* secretion or synthesis by the surface epithelium of the proventriculus in chicks, which may play roles in the defense against pathogenic invasions.

3. Effects of probiotics on the expression of antimicrobial peptides and cytokines in response to stimulation by *Salmonella Minnesota* lipopolysaccharides in the proventriculus and cecum of broiler chicks

Although probiotics-feeding alone did not affect the expression of *AvBDs* in the above study, the efficiency of probiotics-feeding in enhancing the expression of antimicrobial peptides and cytokines in response to pathogenic agents remains unknown. The aim of this study was to determine whether probiotics-feeding affected the expression of *AvBDs*, cathelicidins (*CATHs*) and proinflammatory cytokines in the proventriculus and cecum and the localization of ir-AvBD12 in the proventriculus of broiler chicks challenged with *Salmonella Minnesota* lipopolysaccharides (LPS). One-day-old male 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 µg LPS (1-LPS), or 100 µg LPS (100-LPS) (n = 5 in all groups) in Experiment 1, and with no LPS and 1 µg LPS (n = 6 in all groups) in Experiment 2. Five hours after LPS challenge, the proventriculi and ceca were collected to analyze the expression of *AvBDs*, *CATHs*, Toll-like receptors (*TLRs*) and proinflammatory cytokines expression by reverse transcription-PCR (RT-PCR). In addition to a total of 7 *AvBDs* in the proventriculus and 8 *AvBDs* in the cecum, four *CATHs* (*CATH1*, 2, 3 and 4) were identified in the proventriculus and cecum of chicks. All *TLRs* and proinflammatory cytokines (interleukin (*IL*) 1β, *IL6*, interferon (*IFN*) γ, and tumor necrosis factor superfamily 15 (*TNFSF15*)) were also identified in these organs. There were no histological abnormalities in response to LPS stimulation in non-P-group and P-group. The density of ir-AvBD12 in the surface epithelium of proventriculus was increased in response to 1-LPS and 100-LPS stimulation in the P-group. In Experiment 1, the expression of 2 *AvBDs* in the proventriculus and 6 *AvBDs* in the cecum of 1-LPS chicks was higher in P-group than in the non-P-group. In Experiment 2, the expression of *AvBD1* in proventriculus and 5 *AvBDs* in cecum of 1-LPS chicks was higher in P-group than in non-P-group. Challenge with 100-LPS did not cause differences in the *AvBDs* expression between P- and non-P-group. Expression of *CATHs* in cecum of 1-LPS chicks was higher in P-group than in non-P-group. Although *IL1β* expression was not affected, the expression of *IL6* and *TNFSF15*

in the proventriculus and expression of *IFN γ* in the cecum was lower in P-group than in non-P-group challenged with 100-LPS. These results suggest that probiotics-feeding may enhance the immunodefense system mediated by *AvBDs* and *CATHs* but not by cytokine, which is induced by LPS of *Salmonella* bacteria.

4. Effects of probiotics on the expression of antimicrobial peptides and proinflammatory cytokines in response to stimulation by *Campylobacter jejuni* lipopolysaccharides in the proventriculus and cecum of broiler chicks

It was found that probiotics-feeding enhanced the response to *Salmonella* LPS for the induction of *AvBDs* and *CATHs* expression in the above study. However, it is unknown this effects of probiotics can be expected in the challenge with different LPS from other bacterium than *Salmonella*. The aim of this study was to determine whether probiotics-feeding affected the expression of *AvBDs*, *CATHs* and proinflammatory cytokines in response to *Campylobacter jejuni* LPS (cLPS) challenge in the proventriculus and cecum of chicks. One-day-old male 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 cLPS (0-cLPS), 1 μ g cLPS (1-cLPS), or 100 μ g cLPS (100-cLPS) (n = 6 in all groups) in Experiment 1, and with no cLPS and 1 μ g cLPS (n = 6 in all groups) in Experiment 2. Five hours after cLPS challenge, the proventriculi and ceca were collected to analyze *AvBDs*, *CATHs* and proinflammatory cytokines expression by real-time PCR. In Experiment 1, the expression of *AvBD12* in the proventriculus of 100-LPS chicks was higher in P-group than in the non-P-group. In Experiment 2, the expression of *AvBD4* and *6* in the proventriculus and *AvBD2*, *4*, *6* and *7* in the cecum of 1-cLPS chicks was lower in P-group than in non-P-group. Expression of *CATH1* in the proventriculus, and *CATH1* and *3* in the cecum of 1-cLPS chicks was lower in P-group than in non-P-group. Although *IL1 β* expression was not affected, the expression of *IL6* and *TNFSF15* in the cecum and expression of *IL6* in the proventriculus was higher in P-group than in non-P-group challenged with 1-cLPS chicks. These results suggest that probiotics-feeding partially modulated the immune responses carried out by *AvBDs* and *CATHs* in response to *Campylobacter* LPS in a different manner than *Salmonella* LPS. The different responses by *AvBDs* and *CATHs* may indicate that probiotics-feeding immunomodulation may go either ways by enhancing or inhibiting the innate immune molecules according to the pathogen nature.

5. Conclusion

This study has identified the innate immunodefense functions carried out by tight junction, *AvBDs*, *CATHs* and proinflammatory cytokines in the gastrointestinal tract of broiler chicks and the effects of probiotics-feeding in modulating these functions. The expression of claudin 1 and 5 was upregulated by

probiotics-feeding in the colon of 7-days-old broiler chicks. Probiotics-feeding alone may not affect the expression of *AvBDs* in the proventriculus and cecum, but it may affect the secretion of AvBD12 from the surface epithelium of the proventriculus. Moreover, the effects of probiotics on enhancing the expression of *AvBDs* and *CATHs* in response to stimulation by *Salmonella Minnesota* LPS are also suggested. However, this effect of probiotics in enhancing the innate immune molecules expression was not found in the chicks challenged with *Campylobacter* LPS. The results of the current study suggest that the enhanced expression of the tight junction protein by probiotics-feeding in the colon is beneficial in the protection against pathogenic invasions. Moreover, probiotics-feeding enhanced the expression of *AvBDs* and *CATHs* in the proventriculus and cecum in response to *Salmonella* LPS, but not to *Campylobacter* LPS suggesting a stronger role of probiotics in protection against *Salmonella* infection.