EFFECT OF PROBIOTICS OR HIGH INCUBATION TEMPERATURE ON GENE EXPRESSION AND CELL ORGANIZATION IN THE SMALL INTESTINE AND YOLK SAC OF CHICKS

by

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Dissertation submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Animal and Poultry Sciences

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Sep 23, 2021

Blacksburg, VA

Key words: probiotic, high incubation temperature, epithelial cells, tight junction, small intestine, yolk sac, chicken

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ABSTRACT

(FOR GENERAL AUDIENCE)

The small intestine and yolk sac are important organs for nutrient absorption in hatched chicks or embryonic chicks. These organs also serve as a barrier to prevent pathogens from entering the blood circulation. Intestinal epithelial cells along the villi renew rapidly by proliferation and differentiation. In this research, probiotics which are also known as direct fed microbials temporarily increased expression of the proliferating cell marker Ki67 in the jejunum of healthy young chicks, which suggests that probiotics promote intestinal epithelial cell proliferation. However, probiotics transiently decreased expression of an antimicrobial peptide, which may reduce immune protection in the gut. The yolk sac can also express tight junction proteins. The expression of tight junction proteins was affected by elevated incubation temperature in broiler embryos, which might be related to low hatchability of eggs exposed to heat stress. Avian defense peptides and pathogen recognition receptors were expressed in the YS, which implied that the yolk sac contained an innate immune function. The expression pattern of avian defense peptides was affected by breed (broilers and layers), while the expression level of avian defense peptides was greater in layers than broilers. In summary, the small intestine and the yolk sac are multifunctional organs. Their cell composition, structural integrity, and secretion of antimicrobial peptides can be

affected by environmental factors, such as probiotic supplementation or high incubation temperature.

Effect of probiotics or high incubation temperature on gene expression and cell organization of the small intestine and yolk sac of chicks

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ABSTRACT

The small intestine and yolk sac (YS) are important organs for nutrient absorption and innate immunity in chickens during the post-hatch or prehatch periods. These organs share a similar structure of epithelial cell-lined villi with tight junctions between adjacent cells. Probiotics have been reported to improve chicken growth performance and gut health including promotion of intestinal morphology. However, there are few studies that show the effect of probiotics on ontogeny of intestinal epithelial cells and antimicrobial peptides, or intestinal integrity in young healthy chicks. Heat stress during incubation was shown to increase mortality and decrease hatchability of chicks, while no studies have investigated the effect of heat stress on the integrity of the YS, which might be related to hatching performance. There were four studies conducted in this research: 1) a comparison of the effect of two probiotics on the ontogeny of small intestinal epithelial cells in young chicks; 2) the effect of two probiotics on mRNA abundance of tight junction proteins in the small intestine of young chicks; 3) the effect of high incubation temperature on mRNA abundance of tight junction proteins in the YS of broiler embryos; and 4) comparison of avian defense peptide mRNA abundance in the YS of broilers and layers. In study 1, Probiotics transiently decreased body weight gain (BWG) from day 2 to day 4, but did not affect body weight (BW) from day 2 to day 8, and small intestinal weight and intestinal morphology from day 2 to

day 6. Probiotics did not affect marker gene expression of intestinal stem cells (Olfm4) and goblet cells (Muc2) in all small intestinal segments, but did increase expression of a marker gene of proliferating cells (Ki67), and decreased an antimicrobial peptide (liver-enriched antimicrobial peptide 2, LEAP2) in the jejunum at day 4. Probiotic 1 decreased PepT1, a marker of enterocytes in the duodenum at day 4. These results suggest that probiotics did not improve growth performance and intestinal morphology in young healthy chicks, but temporarily promoted intestinal epithelial cell proliferation and decreased LEAP2 antimicrobial peptide expression in the jejunum. In situ hybridization (ISH) showed that $Ki67^+$ proliferating cells were mainly located in the crypt region and the blood vessels of villi. In study 2, Probiotic supplementation to newly hatched chicks for less than one week did not affect mRNA abundance of the tight junction proteins in the small intestine. Occludin (OCLN) mRNA, which was detected by ISH to be expressed in intestinal epithelial cells in both the villus and crypt regions, was greater in the duodenum of female chicks than males. In study 3, high incubation temperature starting from embryonic day 12 (E12) affected mRNA abundance of the tight junction proteins in the YS, including increased zonula occluden 1 (ZO1) at E13, increased junctional adhesion molecule A (JAMA) and heat shock protein 90 (HSP90) at E17, but decreased tight junction protein JAMA at E19 and OCLN at day of hatch (DOH). These results showed that the YS tight junction proteins were increased by short term heat exposure but decreased by long term heat exposure. In study 4, the expression of avian β defensin 10 (AvBD10), CATHs and toll-like receptors in the YS was examined. Toll-like receptors were highly expressed in the YS at early incubation stages (E7), while CATHs showed a peak expression from E9 to E13, which was similar to the expression pattern of AvBD10. CATHs and AvBD10 mRNA temporal expression patterns were similar in broilers and layers, while their expression levels were different. Layers, especially brown layers, had greater mRNA abundance

for antimicrobial peptides such as AvBD10, CATH1, and CATH2 in the YS. These results demonstrate that the antimicrobial peptide temporal expression patterns in the YS are not affected by breed, but their expression levels are affected by breed. In summary, the small intestine and the YS are essential for nutrient uptake, innate immunity, and maintenance of integrity. The ontogeny of intestinal epithelial cells, such as proliferating cells can be modulated by probiotic supplementation. Similar to the small intestine, the YS can also express tight junction proteins, which can be affected by high incubation temperature. Antimicrobial peptide expression in the intestine of healthy young chicks is also transiently decreased by probiotic supplements. Avian defensin and cathelicidin expression patterns in the YS were not affected by breed.

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Acknowledgements

Dr. Wong: Thank you so much for providing me the opportunity to be a PhD student in your lab. You are so nice and patient to encourage me to anticipate the discussion during lab meetings, to speak louder in public presentations, and to express my thoughts regarding any projects in our lab. Without your help, I could not be calm and relaxed when I do a presentation. I also appreciate that you gave me good suggestions on my projects. From you, I learned how to be a good advisor. Sometimes I took things for granted. You did not directly tell me my thought was wrong, instead you asked me some questions and let me further think about them. In this way, I could deeply analyze my thoughts on research. Also, thank you for patiently revising my writing of project reports and my dissertation. From you, I know how to be a better academic writer and how to tell a good story.

Dr. Corl: I am so glad to have taken your animal nutrition course. I had no background in animal nutrition before. In your class, I gained a deeper understanding about animal nutrition and the opportunity to participate several times in group discussions and group presentations, which leaves me with a good memory about courses in Blacksburg. In addition, thank you for having your student Gabriela do experiments in our lab. We became friends.

Dr. Dalloul: You are so kind and always make jokes to comfort me when I feel nervous during committee meetings and in your office. I appreciate your suggestions about my project in committee meetings, such as consideration about sex effect on marker gene expression of intestinal epithelial cells, which really helped me a lot. Thanks for asking me questions about poultry gut immunity in my preliminary exam. It made me give more thought about the effect of probiotics on the gut immune system.

Dr. Gilbert: I love your big smiles each time I met you, which comforted me when I felt nervous. I so appreciate that you always patiently answered my statistical questions and gave me good suggestions about my statistical analysis and my project. Also thank you for letting students utilize the equipment in your lab, I spent a lot of time using your microscope to take images of my slides in your lab. Also, thanks for your quick responses to my emails.

Dr. Dunnington and Dr. Bradford: Thank you for providing me the opportunities for being a graduate teaching assistant in your genetics course. You showed me how to be a good teacher, and how professional animal breeding specialists look like.

Sasha: Thanks for your assistance and patience for supporting my enrollment and resolving my various problems each time. You are so kind to provide suggestions to limit my expenses.

Dr. Mallory White: Thanks for your kindness and lending me your immunology notes when I prepared for my preliminary exam. Also, thanks for your help when I used the qPCR machine in your lab.

Dr. Yang Xiao and Dr. Jing Zhu: Thanks for your assistance for the utilization of the microscope and plate reader, respectively.

In addition, I would like to thank all the graduate students and undergraduate students in our lab. They helped me collect samples for my project, asked me questions and gave me comments about my presentations. They are so kind and allowed me to borrow their course notes. This really helped me a lot for my course grades. I also had a good time with them in our lab.

Finally, I prefer to thank all the members in my family, especially my husband MengMeng Li. He encouraged me to apply for the PhD position. Without him, I could not try to be a PhD student. To my parents and my parents-in-law, thanks for your help with taking care of my kid during my PhD period even though Blacksburg is far away from our home town. Without you, I may not finish

my PhD program. I would also thank my son, Ethan. He is the sunshine to brighten my heart when I felt tired and he made me happy when I felt frustrated.

Chapter I Literature review

Chicken embryonic development

Broilers have been artificially selected for meat production for many years. Chicken is an important meat source for humans along with pork, beef, and lamb. For meat production, broilers are slaughtered between 5 wks and 7 wks of age, at which time they are heavy enough for commercial sale. Thus, embryonic development, which accounts for more than 33% of the entire life span, becomes very important for broilers (Givisiez et al., 2020). Embryonic development starts early before eggs are laid. After fertilization of the ovum by sperm in the infundibulum, the zygote spends 24 hours in the hen's body before the egg is laid, which provides a warm environment for embryonic development (Ridlen et al., 1964). The first cell cleavage occurs in the isthmus around five hours after fertilization, however, this is only an incomplete cell division and occurs on the surface of the germinal disc. After repeated incomplete cleavages, up to 16 or 32 partially separated cells are produced. At this stage, more central cells completely divide into new cells, which form a cellular mass and subsequently changes into the blastoderm. When the egg is laid, the fertile eggs contains an embryo in the gastrulation stages with three different cell layers including endoderm, mesoderm, and ectoderm. However, embryonic development ceases due to the low temperature in the environment (Bradley and Grahame, 1960; Bell et al., 2002).

During the incubation period, embryos develop within the *area pellucida*, which is in the center of the blastoderm. The lateral border of this area and the *area opaca* form the extraembryonic membrane. The normal stages of embryonic development were described by Hamburger and Hamilton (1951) and are therefore called Hamburger Hamilton (HH) stages. Using these criteria, embryonic development is separated into 46 stages, during which certain tissues or

organs develop. However, Hamburger and Hamilton primarily focused on external structure changes and did not describe much about the development stages of the extra-embryonic membranes, which are important for embryonic development. At stage 18, between 65-69 hours of incubation, the amnion is closed. The amnion is filled with amniotic fluid, which protects the embryo from injury during egg turning. The allantoic membrane fuses with the chorion to form the chorioallantoic membrane (CAM) between embryonic day 5 and 6. This membrane contains many blood vessels. The CAM is not only the site for the deposit of excreted materials, but is also an important site for respiratory gas exchange and calcium absorption from the eggshell. It also aids in the absorption of albumen, ions and water reabsorption, and regulation of acid - base homeostasis (Gabrielli and Accili, 2010). Among the four extraembryonic membranes, the yolk sac (YS) is the first to be formed (Patten, 1920). The YS is derived from the extra-embryonic splanchnopleure and extends over the yolk. It connects with the mid-gut via the yolk stalk. However, the yolk content in the YS cannot directly pass into the gut through the yolk stalk to provide nutrients to the embryo due to the constricted umbilicus (Bradley and Grahame, 1960). Instead, the yolk composition can be absorbed by the endodermal epithelium of the YS and transported to the developing embryos by the vitelline circulation in the YS.

The structure and development of the YS

The YS develops from the *area opaca*, which is in the peripheral area of the blastoderm. Compared with the *area pellucida*, the *area opaca* is less transparent due to the larger size and number of intracellular lipid droplets. At the beginning, the *area opaca* consists of 2 layers, the upper (epiblast) and lower layer, which eventually forms the epithelial ectoderm and endoderm of the YS, respectively. As early as 12 hours of incubation, the edge cells, which are specialized extensions of the ectoderm in the *area opaca*, attach to the interior of the vitelline membrane, so

that the blastoderm begins to expand around the yolk (Bellairs and Osmond, 2014). At around stage 4 - 5 (HH staging), mesoderm from the primitive streak migrates peripherally and invades the area opaca (Kessel and Fabian, 1986). At around 2 days of incubation, the vitelline circulatory system is completed, and the circulation of blood between the heart of the embryo and the vitelline membrane begins (Patten, 1920). At this time, the allantoic circulation has not yet formed. Thus, the YS acts as a respiratory organ to provide oxygen that penetrates the eggshell and albumen for chicken embryos until the allantoic circulation is formed (Patten, 1920). Besides this, the YS transports nutrients from the yolk to the blood circulation of embryos. At around embryonic day 3, the vitelline membrane is broken down. The edge cells in the YS pass over the egg yolk equator between embryonic (E) day 5 and E7 (Bellairs and Osmond, 2014). Approximately at E10, the egg yolk is completely enclosed by the YS (Van der Wagt et al., 2020). From E10 to E15, the weight and area of the YS membrane rapidly increase to support the demands of embryonic development and then decrease from E15 to day of hatch (DOH) (Yadgary et al., 2013). At around E19, the YS starts to be internalized into the abdominal cavity. At E20, the YS is completely contained in the embryonic body before the chick hatches.

The YS consists of three germ layers including ectoderm, mesoderm, and endoderm. The ectodermal cells are flat and form the thin outer layer surrounding the yolk. The tall endodermal cells underlying the ectoderm contain multiple yolk spheres and form the inner layer between the ectoderm and the yolk. These cells transport the yolk material to the blood circulation of the embryo. Mesodermal cells and blood vessels extend between the ectoderm and endoderm and this is an important site for erythropoiesis and nutrient transportation (Yoshizaki et al., 2004; Sheng, 2010; Wong and Uni, 2021). However, the mesoderm layer does not exist in the entire YS. The vascularized region of the YS is called the *area vasculosa*, while the area without blood vessels

and mesodermal cells is called the *area vitellina*. The *transition zone* in the center is also non-vascularized, but contains mesenchymal progenitor cells migrating between the endoderm and ectoderm (Bauer et al., 2013).

Both the YS and gut are derived from the splanchnopleure, so that it is not surprising that the morphology of both are similar (Patten, 1920). During the development of the YS, the morphology of the endoderm in the YS changes from smooth to a series of folds and ridges by E4, which increases the surface area for nutrient absorption (Mobbs and McMillan, 1979; Bellairs and Osmond, 2014). Columnar endodermal epithelial cells (EEC) with intracellular yolk drops form villus-like structures and project into the mass of the yolk. The nuclei of the EEC are located at the basolateral side of the cell. There are also many microvilli randomly distributed in the apical surface of the EEC, which may enhance endocytosis of extracellular yolk granules. In the apical membrane of EEC, there is also some fusion of the plasma membrane between two adjacent EECs, which is called the apical junction. The apical tight junction seals the two adjacent endodermal cells to prevent the yolk content from entering the vitelline circulation and restricts the intercellular passage of macromolecules. However, unlike the typical tight junction, there are large intercellular dilated spaces between the fusions. The reason for the presence of dilations is still unknown (Mobbs and McMillan, 1979).

The function of the YS

Due to its unique structure, the YS is a multifunctional organ. Prior to the formation of the allantoic circulation, the YS acts as a respiratory organ to carry sufficient oxygen to supply the developing embryo through the vitelline circulation (Patten, 1920; Bradley and Grahame, 1960). Besides the respiratory function, the YS plays an important role in nutrient absorption, immunity, and erythropoiesis.

Composition of the egg

Unlike mammals, chicken embryos only obtain nutrients from the egg for embryonic development during incubation. The eggshell is the main source of calcium, which is important for bone formation. The egg content is affected by egg size, breed, age of the hen, and season, etc. The albumen content varies between 65% -75% of the egg content (Marion et al., 1964; Yadgary et al., 2010). The main component of albumen is water, which accounts for 88% of the total (Marion et al., 1964; Vieira, 2007; Yadgary et al., 2010). The second major component of albumen is protein, which is around 11% of the total. Carbohydrates and minerals are also present in the albumen at lower amounts (Romanoff and Romanoff, 1949). The egg yolk is the richest compartment in chicken eggs, with 48.7% water, 32.6% lipid, 16.6% protein, around 1% carbohydrates, and 1% minerals (Romanoff and Romanoff, 1949). Although the main component of the yolk is also water, the solid content in the yolk accounts for 70.4% of the solid content in the whole egg. The lipid of the egg is only in the yolk.

The composition of the egg content is changed during embryonic development. From E2 to E7, water and chloride ions in the albumen are drawn into the yolk via active transport to form sub-embryonic fluid, which dilutes the condensed yolk content and facilitates nutrient absorption by the YS endodermal cells (Willems et al., 2014). In the second half of incubation, as albumen proteins are moved into the amniotic cavity, the main albumen protein ovalbumin also appears in the yolk. This indicates that albumen absorption may need to be transported by the EEC in the YS (Yoshizaki et al., 2002; Willems et al., 2014).

Nutrient digestion and absorption in the YS

Lipid metabolism in the YS

Lipid is the majority of the dry matter in the yolk and is present in the form of lipoprotein. Low-density lipoprotein accounts for 68% of the total lipids (Anton, 2007). Gene expression profiling in the YS by microarray at E2 to E4 showed expression of many genes involved in lipid metabolism, including fatty acid binding protein FABP2, phospholipases, monoacylglycerol acyltransferase, long chain acyl-CoA synthetase, and apolipoproteins. This demonstrates the capacity of the YS to take up, break down, repackage, and transport lipids into the extra-embryonic circulation (Nakazawa et al., 2011; Sheng and Foley, 2012). The lipoprotein in the yolk can be engulfed or phagocytosed by the EEC or endocytosed by the EEC receptor complex, LRP2-cubilin-amnionless in the *area vasculosa* (Speake et al., 1998; Bauer et al., 2013). Lipase was also detected in the YS, which suggests that lipoproteins can also be hydrolyzed by lipase and absorbed by the EEC (Yadgary et al., 2013). Bile acid also is present in the YS membrane and the yolk content, which facilitates the digestion of lipid. However, the origin of the bile is still unknow, it might be synthesized by the YS or come from the liver through the yolk stalk (Yadgary et al., 2013).

Proteomic analysis of the egg yolk at E0, E10, and E18 showed the presence of some differentially expressed proteins, such as vitellogenin and apolipoprotein, which are involved in lipid transportation and localization (Zhu et al., 2020).

Protein and amino acid absorption in the YS

Free amino acids are present in the yolk of fertile eggs. Based on metabolomic analysis, the amount changed during incubation (Fitzsimmons and Waibel, 1968; Liu et al., 2021). Yadgary et al. (2011) reported that mRNA abundance of aminopeptidase N (APN) in the YS increased from E11 to E17, but declined from E17 to E20, which indicates that the capacity for digestion of yolk peptides peaked around E17. This result partially coincided with the expression of the oligopeptide

transporter PepT1, whose expression level increased from E11 to E15, and decreased from E15 to E20. Similar results were also found in the study of Speier et al. (2012). This study found that glutamate/aspartate transporter (EAAT3) mRNA abundance in the YS was increased towards hatch. The neutral amino acid transporter B^0AT mRNA had a similar expression pattern in the YS, with the greatest mRNA abundance between E17 and E19 (Speier et al., 2012). However, the cationic amino acid transporter (CAT1) mRNA declined from E11 to E13, and then increased from E15 to E17 (Yadgary et al., 2011). These results suggest that different expression patterns of transporters may be due to their different location (basolateral or apical) in the EEC or the type of amino acids present in the yolk (Yadgary et al., 2011).

Carbohydrate metabolism in the YS

Together with the liver, the YS plays an important role in glucose metabolism during incubation. Yadgary and Uni (2012) evaluated the carbohydrate levels and mRNA abundance of gluconeogenic and glycogenic enzymes in the YS or YS tissue. At the early incubation stages, the small amount of glucose, which decreased from E0 to E11, serves as the energy source for the embryos. From E11 to E19, the amount of glucose increased in the yolk. Key enzymes in the gluconeogenesis pathway, such as fructose 1,6-bisphosphatase (FBP1), phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6PC2), were also expressed in the YS. This indicates that the increase in glucose might come from the gluconeogenesis pathway of the YS tissue. The YS also acts as the main organ for glycogen storage during incubation. The concentration of glycogen in the YS increased from E11 to E19, which coincided with the increased mRNA abundance of key enzymes of glycogen synthesis, including glycogen synthase (GYS2). However, glucose and glycogen concentrations in the yolk decreased from E19 to DOH, because they were used during the hatching process (Yadgary and Uni, 2012).

There are many glucose transporters expressed in the YS, which uptake glucose from the yolk to the vitelline circulation and transport the glucose to the developing embryo. Yadgary et al. (2011) and Speier et al. (2012) showed that the mRNA abundance of the sodium glucose transporter SGLT1 increased during incubation. The fructose transporter GLUT5 mRNA was also expressed in the YS tissue, with its expression level decreasing over time (Speier et al., 2012). An in situ hybridization study found that SGLT1 mRNA was distributed in the endodermal cells of the YS (Zhang et al., 2019a).

Erythropoiesis

The role of erythropoiesis in the YS was reviewed by Sheng (2010). The YS is the only site for erythropoiesis between E1 to E5. As early as stage HH6, blood islands appear in the mesodermal expansion of the extra-embryonic membrane. Hemoglobin is initially expressed in the peripheral blood islands. Some of the blood island cells differentiate into blood cells, and some differentiate into endothelial cells. After formation of the circulation, the blood cells are changed from blood clusters to free circulating cells. Due to the expression of hemoglobin and the immature morphology, blood cells are still called primitive erythrocytes until E5, when definitive erythrocytes appear in the blood vessels. By E9, primitive erythrocytes are completely matured into definitive erythrocytes. In the meantime, the formation of bone marrow starts, which will become the main erythropoiesis sites from E12. Guedes et al. (2014) also verified the erythropoiesis function of the YS using histological analysis. Hematopoietic foci were enhanced from E3 to E13 and decreased from E14 to E19 and E20. They also demonstrated the granulopoietic function of the YS, which generates mature leukocytes in the extravascular compartment of the YS.

Immune function of the YS

Bacteria in eggs

Eggs are normally in a non-sterile environment during egg formation and after oviposition. Despite raising of SPF (specific pathogen free) chicken hens, bacteria such as *Flavobacterium* and *Lactobacillus* naturally exist in the magnum, which is the site for albumen formation. A large diversity of bacteria is present in the cloaca of SPF hens (Lee et al., 2019). This provides the opportunity for penetration of bacteria into the egg content. Lee et al. (2019) reported that bacteria in the egg white predominantly colonized the embryonic gut at E18, which suggests that before being exposed to the non-sterile environment, embryos obtained bacteria from the albumen. In addition, their results suggest that bacteria in the oviduct of SPF hens can be vertically transferred to the egg white during egg formation. After the egg is laid, bacteria from the environment such as cages and floor pens also have a chance to colonize the eggshell, which might also penetrate into eggs via horizontal transmission (Gantois et al., 2009). However, most fertile eggs are still viable, which is due to the natural defense mechanisms in the eggs (Hincke et al., 2019).

Defense mechanisms in eggs

In fertile eggs, the eggshell and egg white not only act as physical or chemical barriers, but also contain antimicrobial peptides or lysozyme to prevent the entrance of non-specific pathogens. The egg yolk contains immunoglobin Y (IgY) which is derived from the maternal hen and is transported by the YS into the embryonic circulation to provide immunity in the egg. As the chicken embryonic immune system develops, immune cells such as dendritic cells, natural killer (NK) cells generated from bone marrow, and $\gamma\delta$ T cells generated from the thymus, migrate to the embryonic spleen, intestine or peripheral lymphoid to protect the embryo from pathogens (Alkie et al., 2019). In addition, cytokines such as IFN-γ and interleukins are also expressed in the

embryonic spleen as early as E12 (Abdul-Careem et al., 2007). Toll like receptors (TLRs), which induce the innate immune response by recognition for pathogen-associated molecular patterns (PAMPs) were identified in whole embryos (Kannaki et al., 2015).

Avian host defense peptides

Host defense peptides (HDP) are also called antimicrobial peptides, due to their ability to kill a wide spectrum of bacteria (Cuperus et al., 2013; Zhang and Sunkara, 2014). The properties of HDP including their structures or functions have been reviewed in detail (Cuperus et al., 2013; Zhang and Sunkara, 2014). HDP are small cationic peptides existing in both vertebrates and invertebrates. They have the ability to permeabilize bacterial membranes, induce the disruption of bacterial membranes, and subsequently kill bacteria (Hollmann et al., 2018). Besides the antimicrobial activity against Gram-positive and Gram-negative bacteria, HDP also have function against viruses and fungi, and act as immunomodulators to regulate immune responses to infection and inflammation, or to participate in the wound-healing process (Hilchie et al., 2013; Findlay et al., 2016). In avians, there are three major families of HDPs including avian β defensins, cathelicidins, and liver-enriched antimicrobial peptide 2 (LEAP2), which are expressed in the chicken embryos as early as E3 (Meade et al., 2009).

Unlike mammals, in which there are three sub-families including α, β, θ defensins, only β defensins are found in avians, which include 14 members. Avian β defensins (AvBDs) are short cationic antimicrobial peptides that consist of less than 100 amino acids (Wong and Uni, 2021). Genes encoding the AvBDs are located on chromosome 3 (Lynn et al., 2007). The mature AvBDs have a conserved structure of β - sheet and three disulfide bridges between two cysteine residues (Cys^1 - Cys^5, Cys^2 - Cys^4 and Cys^3 - Cys^6) (Klotman and Chang, 2006). The AvBDs play an important role in innate immunity of birds against bacteria, viruses, and parasites in the gut and

reproductive systems. However, the distribution and expression of the various AvBDs differ (Cheng et al., 2015). In embryos, AvBD expression was regulated by age. AvBD2, AvBD6, and AvBD7 mRNA in whole embryos increased from E3 to E6, followed by a decrease from E6 to E9. However, AvBD9, AvBD10, and AvBD14 mRNA in whole embryos increased from E3 to E9. AvBD10 and AvBD14 also had a greater expression level at E12 in the abdomen and head respectively. These results suggest that AvBDs are also expressed in the embryonic body and provide a defense mechanism at different developmental stages.

Cathelicidins (CATHs) also have antimicrobial activity against a broad spectrum of bacteria including antibiotic resistant strains and an anti-inflammatory function by inhibiting expression of proinflammatory genes (Xiao et al., 2006). The structures of CATHs have a conserved cathelin domain, which is where the name is derived (Zanetti et al., 1995). Genes encoding CATHs are located on chromosome 2. However, only CATH1 and CATH3 have high sequence homology (>70%), with no obvious sequence homology between CATH1/CATH3 and CATH2 or CATHB1 (Veldhuizen et al., 2013). The distribution of CATHs also differ. CATH1, CATH2, and CATH3 were expressed in heterophils of the intestine and three immune organs, while CATHB1 was exclusively expressed in the epithelial cells of the bursa of Fabricius (Goitsuka et al., 2007; Li et al., 2020). CATHs were also expressed in chicken whole embryos (Meade et al., 2009). CATH1, CATH2, and CATH3 mRNA in whole embryos increased from E3 to E6, decreased from E6 to E9, and increased again from E9 to E12. CATHB1was not expressed in embryos at E3 and E6, but was expressed at E9 and E12.

LEAP2 is a short 76 amino acid antimicrobial peptide against bacteria and parasites (Su et al., 2017). The LEAP2 gene contains three exons and is located on chromosome 13. LEAP2 is expressed in epithelial cells of many organs including liver, small intestine, lung, and kidney

(Townes et al., 2004). LEAP2 was also expressed in chicken whole embryos, with high expression from E6 to E8, and lower expression at other embryonic stages (Michailidis, 2010). However, LEAP2 expression level was very low in the YS (Zhang and Wong, 2019).

AvBD mRNA in the YS

The YS also plays an important role in immune function. Besides transportation of maternal IgY from egg yolk to embryos, it also generates $CD45^+$ cells as early as E2 and produces macrophages during the embryonic period (Balic et al., 2014; Dóra et al., 2017). In addition, like other extra-embryonic membranes, the YS also plays an essential role in innate immunity as a physical barrier and by expression of antimicrobial peptides. AvBDs were expressed in the YS (Zhang and Wong, 2019). The mRNA abundance of AvBD 1, 2, 7, and 10 were greater than other AvBDs. Their expression patterns showed an increase from E7 to the middle incubation period (E9 to E13), and a decline towards E19. A combination of Giemsa staining and RNAscope in situ hybridization demonstrated that AvBD1, 2, and 7 were expressed by heterophils in blood vessels of the YS. AvBD10 had the greatest expression level compared with other AvBDs, which suggests that it plays an important role in the defense function of the YS. In situ hybridization showed that AvBD10 mRNA was expressed by EEC of the YS, however, how it supports the immune system in eggs is still unclear.

Regulation of thyroid hormones

Before the thyroid gland in the embryo is functional, the YS may play an important role in the regulation of thyroid hormones. The YS transfers the thyroid hormones (TH) from the yolk content by expression of the TH transmembrane transporters (Too et al., 2017). Yadgary et al. (2014) found that TTR, the blood transporter transthyretin, was highly expressed in the YS from E13 to E17, but did not determine its expression at earlier stages. TH-inactivating iodothyronine

deiodinase 3 (DIO3) had greater expression in the YS at E5 and E10, and lower expression from E11 to E21. The YS also expressed thyroid hormone regulator genes (Too et al., 2017). TH-activating deiodinase 2 (DIO2) had a lower mRNA abundance from E4 to E13, and greater expression level from E14 to E21. However, the expression pattern of DIO2 was not consistent with the finding in the study of Dayan et al. (2020), which might be due to the chicken breeds used (Wong and Uni, 2021). Taken together, the YS is not only responsible for the transport of TH but also regulates the activity of TH.

Effect of heat stress on the embryo and YS

Long term heat stress at early stages (E1 - E9) can cause lethal and teratogenic effects on the embryo (Peterka et al., 1996). Continuous heat stress (24 hours /day at 39.5 ℃) from E7 to E16 decreased the hatchability of broilers, and body weight and quality of newly hatched chicks, which had non absorbed YS or rough navels (Piestun et al., 2008). The YS utilization was also affected by long term elevated temperature incubation (39.3 ℃ from E0 to E18) resulting in a large residual yolk at day of hatch (Dayan et al., 2020). However, intermittent heat stress (12 hours at 39.5 ℃) did not affect hatchability, and body weight and quality of newly hatched chicks (Piestun et al., 2008). The intermediate response to heat stress at the molecular level also was observed in the gastrocnemius muscle (Naraballobh et al., 2016). The expression of genes involved in lipid metabolism and fatty acid oxidation (FABP1 and PPARA), and energy metabolism (PPARGC1) was increased by exposure to high incubation temperature (38.8 ℃) from E7 to E10. The mitochondrial respiratory activity was also increased in the leg muscle by early (38.8 ℃, E7 - E10) and in the breast and leg muscle by late (38.8 ℃, E10 - E13) heat stress exposure (Krischek et al., 2016).

As mentioned above, high incubation temperature (39.3 ℃ from E0 to E18) decreased yolk utilization, especially during the later stages from E18 to DOH. At the same time, expression of genes involved in nutrient metabolism and TH regulation were also affected by heat stress (Dayan et al., 2020). Regression analysis showed that low-density lipoprotein receptor (LRP2), which is involved in lipid uptake, was increased from E5 to E11 and decreased from E15 to E19, while in the controls, there was a linear increase from E5 to E19. The expression pattern of ApoA1, a marker of lipid metabolism, changed from a quadratic polynomial fit to a moderate linear fit. These results imply that both lipid transportation and metabolism were reduced by elevated incubation temperature, which also corresponded with the decrease of yolk utilization. Similar results were also found for the expression of the oligopeptide transporter PepT1 mRNA, which decreased from E15 towards hatch in the heat stress group, but remained constant in the control. The expression pattern of glycogen synthase GYS2 was also altered by heat stress during incubation from a polynomial fit to no change in expression. Thus, heat stress reduces yolk utilization by decreasing lipid and peptide transport from the yolk to EEC and a decline of lipid metabolism and glycogen storage.

The regulation of TH by the YS was also affected by heat stress. Triiodothyronine T3 is one of the hormones in the hypothalamus-pituitary-thyroid axis, which is involved in regulation of body temperature in chickens. T3 is present in the yolk, which comes from the maternal hens, and is regulated by the inactivator and activator expressed in the YS (McNabb, 2007). In broilers, the expression pattern of DIO2, an activator of TH that is responsible for deiodination of T4 to T3, fit a quadratic polynomial regression in the control with a rapid decrease towards hatch. However, its expression showed a slower decrease towards hatch in the high incubation temperature group. (Dayan et al., 2020). This suggests that the regulation of TH in the YS might also be affected by

heat stress, which may contribute to the regulation of embryonic temperature during the heat exposure period.

Structure and function of the small intestine

The formation of the YS from the extra-embryonic splanchnopleure is accompanied by the formation of the embryonic gut from the intra-embryonic splanchnopleure. This helps explain the high similarity of structure and function between the YS and the gut (Patten, 1920; Wong and Uni, 2021). The epithelium, derived from endoderm, undergoes transformation from a flat surface to villi before hatch. The gut mesenchyme from the splanchnic mesoderm develops into a layer consisting of smooth muscle and neurons, which controls coordinated peristalsis (Huycke and Tabin, 2018). Unlike mammals, the structure of the small intestine in chickens only consists of three layers: mucosa (innermost), muscularis, and serosa (outermost), without a submucosa layer (Gabella, 1985). The mucosa is the most complex layer in structure and function and is divided into three layers including epithelium, the lamina propria, and muscularis mucosae. Small intestinal epithelia project into the gut lumen to form the villi, which increase the surface area and access to nutrients. There is microvasculature in the lamina propria to transport the absorbed nutrients into the circulatory system (Shini et al., 2021).

The small intestine is the primary site for feed digestion and nutrient absorption from the pylorus to the ileo-cecal-colon junction. It is divided into three segments in chicken: duodenum, jejunum, and ileum. The duodenum is the upper segment of the small intestine, which is also known as the duodenal loop, which ends at the entrance of the pancreatic and bile duct. The jejunum is the middle segment, which is separated from the ileum by Meckel's diverticulum (the residual yolk stalk) (Noy and Sklan, 1995; Svihus, 2014). The duodenum is the site for partial digestion of nutrient including starch, protein, and fat due to the entry of digestive enzymes from

the pancreas and bile secreted by the gall bladder (Riesenfeld et al., 1980). The jejunum is the continual site for nutrient digestion and the major site for nutrient absorption, including fat and fatty acids, carbohydrates, and proteins (Del Alamo et al., 2009; Svihus, 2014; Tancharoenrat et al., 2014; Rodriguez-Sanchez et al., 2019). The ileum plays an important role in absorption of water and minerals (Svihus, 2014).

Microbiota in the small intestine

As early as the embryonic stages, microbes colonize the gut of broiler chickens, which originate mainly from the maternal oviduct (Lee et al., 2019). In the first week after hatch, commensal microbes rapidly colonize the gastrointestinal (GI) tract of chickens due to exposure to the environment with a diverse microbiota (Ballou et al., 2016; Richards et al., 2019). The microbiota in the chicken intestine mainly consists of various bacteria, with lower levels of fungi, virus, and methanogenic archaea (Xiao et al., 2017; Yadav and Jha, 2019). Although the microbiota community in the small intestine changes over time, recent studies reported that *Lactobacillus* was the predominant genus in the duodenum, jejunum, and ileum of broilers at all ages (Xiao et al., 2017; Glendinning et al., 2019; Liao et al., 2020). Besides *Lactobacillus*, *Enterococcus* and *Corynebacterium* also were highly abundant in the small intestine of 42 day-old broilers (Xiao et al., 2017). However, Lu et al. (2003) reported that *Lactobacillus (70%)*, *Clostridiaceae (11%)*, *Streptococcus (6.5%)*, and *Enterococcus (6.5%)* related sequences were the majority of the total sequences detected in the ileum. The bacterial community in the ileum were transient at day (D) 3, which would become stable from D7 to D21, and between D21 to D28 (Lu et al., 2003). Bacterial density is also affected by location. Compared to the small intestine, there was greater abundance and diversity of bacteria in the cecum of broiler chicken (Shapiro and Sarles, 1949; Salanitro et al., 1974; Xiao et al., 2017). The duodenum contained the lowest bacterial

density compared with the ileum, ceca and colon because of the short transit time, dilution by digestive juices and low pH, which are less favorable for bacteria (Shapiro and Sarles, 1949; Rehman et al., 2007; Shang et al., 2018). However, Xiao et al. (2017) reported that the microbiota diversity in the duodenum is the greatest compared with the cecum, jejunum and ileum. This might relate to the function of the different segments of the intestine.

Commensal bacteria in the gut of chickens play a role in pathogen exclusion by competitively adhering to the intestinal wall. These bacteria are also capable of stimulating the immune system and enhancing gut development, which may be related to the production of short chain fatty acids (SCFA) (Han et al., 2016; Clavijo and Flórez, 2018; Shang et al., 2018). Most intestinal commensal bacteria have the ability to ferment undigested carbohydrates such as fibers into SCFA. SCFA act as an energy source for enterocytes and also stimulate the immune system by increasing mucin production and expression of antimicrobial peptides (Tellez et al., 2006; Sunkara et al., 2012). SCFA can also promote enterocyte proliferation and improve the intestinal barrier by increasing expression of tight junction proteins (Tellez et al., 2006; Peng et al., 2009). Despite the low density of microbes in the small intestine, fermentation also occurs in the small intestine, which provides a low level of SCFA to the small intestinal cells. Total SCFA and acetic acid were linearly increased by age in the ileum from D1 to D42, while there was no significant changes in the concentration of total SCFA in the duodenum and jejunum (Liao et al., 2020). Isobutyric acid and isovaleric acid were detected in the ileum over the whole growth period. However, isobutyric acid and isovaleric acid were only detected in the jejunum from D7 to D21, or from D1 to D14, respectively. Isobutyric acid was not detected in the duodenum, while isovaleric acid was detected in the duodenum from D21 to D42. There was no propionic acid, butyric acid, and valeric acid detected in the small intestine from D1 to D42. However, research

on commensal bacteria and SCFA mainly focused on the ileum and ceca, and the relationship of SCFA and development of the duodenum and jejunum is still unclear (Liao et al., 2020). The microbial community in the small intestine could be modulated by supplemental additives such as probiotics.

Effect of probiotics on the small intestine

Probiotics are defined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989), which is also referred to as direct feed microbials (DFM). As the demand for antibiotic elimination in the poultry industry increases, the use of probiotics as feed additives has increased over the years (Jha et al., 2020). Besides beneficial bacteria, probiotics also consist of fungi and yeast. The common genera of probiotics used in poultry include lactic acid bacteria (*Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*), *Bacillus*, and yeast such as *Candida* (Park et al., 2016; Jha et al., 2020). However, due to the strain-specific biological activity of probiotics, it is important to identify the proper strain that meets a series of requirements. The characteristics of probiotics include non-pathogenicity, resistance to gastric acid and bile, capability of adhesion to intestinal mucus or epithelial cells, inhibition of adhesion and colonization of enteric pathogens, enhancement of viability of beneficial bacteria, and having beneficial effects on host animals (Fijan, 2014; Clavijo and Flórez, 2018).

Probiotics have been widely used as feed supplements in the poultry industry, which play an important role in modulation of intestinal microbiota by competitive exclusion of pathogens, nutrient competition, decreasing pH, which is not favorable for pathogens, and producing bactericidal and antimicrobial substances (Pan and Yu, 2014; Clavijo and Flórez, 2018). In addition to this benefit, probiotics can be used as an alternative strategy as growth promoters to

enhance growth performance of broilers via increasing body weight gain (BWG) and reducing feed conversion ratio (FCR). This may involve the alteration of small intestinal morphology by probiotics (Park et al., 2016; Jha et al., 2020). Additionally, probiotics counter enteric pathogenic infections by modulation of the immune response and promotion of the development of the intestinal immune system, and neutralization of enterotoxins (Park et al., 2016; Jha et al., 2020).

Effect of probiotics on growth performance of broilers

Supplementation with probiotics promotes growth performance of broilers, such as increasing body weight (BW), BWG, and feed efficiency. The addition of a probiotic mix of *Lactobacillus fermentum* and *Saccharomyces cerevisiae* for 42 days increased average daily gain (ADG) and feed efficiency of broilers from D1 to D21 (Bai et al., 2013). Zulkifli et al. (2000) also reported that supplementation of a probiotic mixture based on *Lactobacillus* strains for 42 days increased BW at D21 and D42, BWG from D1 to D21 and from D21 to D42, and feed efficiency from D1 to D21. Probiotic supplementation with one of three *Bacillus* strains (*Bacillus subtilis natto*, *Bacillus licheniformis*, or *Bacillus cereus*) for 42 days also increased BW and daily weight gain from D1 to D21 and from D21 to D42, while it did not change the FCR (Gong et al., 2018). Zhang et al. (2021) also reported that a probiotic supplement with one of the three lactic acid bacteria (*Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacterium*) increased the BW of female broilers at D42. Meanwhile, addition of the probiotics dramatically reduced the *Escherichia coli* and *Salmonella* populations, and increased the total counts of *Lactobacillus* at D21 or D42. This indicates that probiotics improved growth performance probably by altering the intestinal microbiota (Baldwin et al., 2018).

Probiotic supplementation also improved digestive enzyme activity. Supplementation of *Bacillus* strains increased the total protease, trypsin, amylase, and lipase activities in the duodenal

content of broilers and trypsin and amylase activities in ducks (Rajput et al., 2013; Gong et al., 2018). A similar effect on digestive enzymes was also reported in broilers supplemented with a single strain of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* or a probiotic mixture of *Lactobacillus* strains (Zhang et al., 2021). Increasing digestive enzyme activity may be associated with greater nutrient utilization and enhancement of nutrient absorption, which may improve growth performance (Gong et al., 2018).

Effect of probiotics on small intestinal morphology

Probiotics may improve growth performance of broilers by a change in intestinal morphology such as an increase in villus height (VH) or the villus height/ crypt depth ratio (VH/CD). The intestinal epithelia project into the lumen of the gut and generate intestinal villi, which increase the surface area for nutrient absorption. Thus, the increase in intestinal villi especially in the jejunum, the main site for nutrient absorption, would enhance nutrient uptake, and subsequently promote the growth of broilers. A greater VH/CD implies more surface area for nutrient absorption and lower energy cost for maintenance, which also benefits broiler growth. Li et al. (2019) reported that dietary *Bacillus coagulans* increased VH/CD in the jejunum at D21, which was associated with an increase in BW on D21 and ADG from D1 to D21. Probiotic supplementation with *Lactobacillus fermentum* for 11 days increased VH in the duodenum, jejunum, and ileum at D8 and D11, and BW of broiler chicks on D9, D10, and D11 (Šefcová et al., 2021). These results suggest that probiotics could improve growth performance by promoting changes in intestinal morphology.

Probiotics alleviate or prevent pathogens from impairing intestinal morphology. Šefcová et al. (2021) reported that 4 or 7 day post infection (dpi) with *Campylobacter jejuni* (infected at D4) decreased VH in the duodenum and ileum, with these negative effects reversed by

pretreatment with the probiotic *Lactobacillus fermentum* from D1. In young broiler chicks challenged with *Salmonella* Typhimurium, a diet supplemented with *Lactobacillus* strains increased duodenal, jejunal, and ileal VH and ileal VH/CD along with an increase of BWG from DOH to D10 (Ashayerizadeh et al., 2016). Increasing VH would be beneficial due to an increase in absorptive surface area for nutrient uptake. Thus, these reports suggest that probiotics may ameliorate the harm to intestinal morphology caused by the pathogens. Furthermore, probiotics could also improve intestinal morphology by promoting a wave-like pattern of intestinal villi and preserving intact and densely distributed microvilli, which are absent in antibiotic-treated chicks (Park et al., 2016). Probiotics also increased the surface area that can access nutrients, and subsequently enhance nutrient absorption.

Effect of probiotics on small intestinal integrity

Probiotics play an important role in promotion of intestinal integrity by either increasing expression of tight junction proteins, which reduces intestinal permeability, or decreasing expression of tight junction proteins, which increases intestinal permeability. A challenge with *Salmonella* Typhimurium to newly hatched chicks significantly increased intestinal permeability and decreased mRNA expression of tight junction zonula occludens 1 (ZO1) and claudin 5 (CLDN5), and increased CLDN2 mRNA abundance. CLDN5 is an important tight junction protein member that promotes intestinal integrity and reduces intestinal permeability, while increasing CLDN2 expression reduces intestinal permeability due to its role in inducing cation selective pore expression. Thus, the alteration of tight junction protein expression increased intestinal integrity, but these negative effects of *Salmonella* Typhimurium were alleviated by supplementation with *Lactobacillus plantarum* (Wang et al., 2018). A *Lactobacillus plantarum* supplemented diet also promoted the mRNA expression of tight junction protein CLDN1 in the ileum of layer chickens

on day 3 after challenge with *Clostridium perfringens* (Xu et al., 2020). These results suggest that probiotics have the ability to promote cell integrity that was impaired by intestinal pathogens.

Probiotics also mitigate impairment of intestinal integrity from heat stress. Broiler chickens are susceptible to heat exposure, which results in low body weight gain, damaged intestinal morphology and increased permeability to endotoxin (Bottje and Harrison, 1985; Nanto-Hara et al., 2020). Song et al. (2014) reported that heat stress decreased transepithelial electrical resistance (TER) and increased intestinal permeability, along with a decreased mRNA abundance of tight junction proteins ZO1 and occludin (OCLN). Although a probiotic mixture (*Lactobacillus plantarum*, *Bacillus subtilis*, and *Bacillus licheniformis*) did not increase TER and reduce intestinal permeability, there was an increased mRNA expression of OCLN, which would improve intestinal integrity. However, the effect of probiotics on tight junction proteins only focused on chickens challenged by pathogens or heat stress. There are fewer studies in healthy chicks.

Effect of probiotics on the intestinal immune system

The intestinal immune system plays an important role in preventing pathogens from entering the body circulation of broiler chickens. Beneficial commensal bacteria competitively exclude pathogens, mucin secreted from goblet cells is the primary component of mucus (Rajput et al., 2020). Mucus, which is located between intestinal villi and surrounds the tip of the villi, acts as the first physical barrier to separate intestinal bacteria and the epithelium. Intestinal epithelial cells lining the villi are tightly sealed by junctions between adjacent cells to prevent pathogens from passing through the epithelium. Gut associated lymphoid tissue in the lamina propria underneath the intestinal epithelium is highly populated with leukocytes. There are also some lymphocytes located in the epithelium and sub-epithelium (Bjerregaard, 1975; Lillehoj and Trout, 1996)

Besides maintenance of a healthy microbial balance and competitive exclusion of pathogens, pretreatment with probiotics stimulates the development of the intestinal immune system and immune response against pathogens and prevents inflammation. Supplementation of a commercial probiotic mixture PrimaLac® StarLabs (*L. acidophilus*, *L. casei*, *Enterococcus faecium*, and *Bifidobacterium bifidum*) for 14 days increased mRNA expression of mucin and mucin glycoprotein level in the jejunum of broiler chicks (Smirnov et al., 2005). A probiotic mixture with *L. fermentum* and *Saccharomyces cerevisiae* stimulated the proportion of T cells, T helper lymphoid cells, and T cytotoxic lymphoid cells by increasing TLR2 and TLR4 expression in the foregut of broiler chickens (Bai et al., 2013). Broiler chickens challenged with *Salmonella* had a lower antibody titer against infectious bursal viruses and Newcastle disease virus, while these negative effects were reversed or alleviated by supplementation with the Probiotic Gallipro® containing *Bacillus subtilis* (Sadeghi et al., 2015). In *Clostridium perfringens* challenged chickens, probiotic supplementation modulated the immune response by decreasing mRNA abundance of the pro-inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-17 in the jejunum (Emami et al., 2020). These results suggest that probiotics modulate the immune system to promote intestinal health of chickens.

Factors influencing effectiveness of probiotics

The effectiveness of probiotics in broilers is strain specific, and is also affected by the origin of the beneficial bacteria, the health of the chicken and the time of administration. For example, *Bacillus subtilis* was reported to stimulate growth performance of broilers at later stages from D28 to D41, but did not affect intestinal morphology in the jejunum at any age (Wang et al., 2016a). Probiotic utilization with a mixture that included *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bacillus subtilis*, and *Enterococcus faecium* did not improve BWG from

D1 to D42 but did increase duodenal villus height at D42 (de Souza et al., 2018). Probiotics of animal intestinal origin were shown to better inhibit pathogenic adhesion to the intestinal epithelium (Collado et al., 2005). The effectiveness of probiotics seems greater in pathogen-challenged or heat stressed chickens than in healthy chickens (Sadeghi et al., 2015). Long-term administration of probiotics tended to improve growth performance compared to short-term supplementation. Thus, it is important to identify the specific strains that are of benefit to the poultry industry and use them depending upon specific environmental conditions.

Cellular organization of the small intestinal epithelium

The small intestinal epithelium is composed of a single epithelial cell layer lining the intestinal villi, which extend into the lumen to increase the surface area that interacts with the gut contents. Due to its unique structure and functional epithelial cells, the intestinal epithelium plays an important role in nutrient absorption and acts as a physical barrier to prevent pathogens from entering the body circulation. The small intestinal villi project into the intestinal lumen and form finger like structures that increase the digestive and absorptive surface areas. The base of the villi invaginates towards the mucosal muscle layer and forms tubular indentations, which form the intestinal crypt (Yamauchi, 2002).

The crypt-villi structure of the small intestine can be damaged by mechanical abrasion and other stressors from the gut content (Gehart and Clevers, 2019). Due to the proliferation and differentiation of active intestinal stem cells in the crypt, intestinal epithelial cells in the villus region renew rapidly around every 3 to 5 days (Fernando and McCraw, 1973). Prior to differentiation into mature epithelial cells, intestinal stem cells partially differentiate into progenitor cells, which are also called transit-amplifying (TA) cells (Figure 2-1). In mammals, TA cells differentiate into enterocytes, goblet, enteroendocrine, and Tuft cell, which migrate towards

the tip of the villi to renew sloughed off epithelial cells. Paneth cells stay in the crypt. In chickens, the organization of the small intestinal epithelial cells is similar to that in mammals, but there still are some differences (Zhang et al., 2019b).

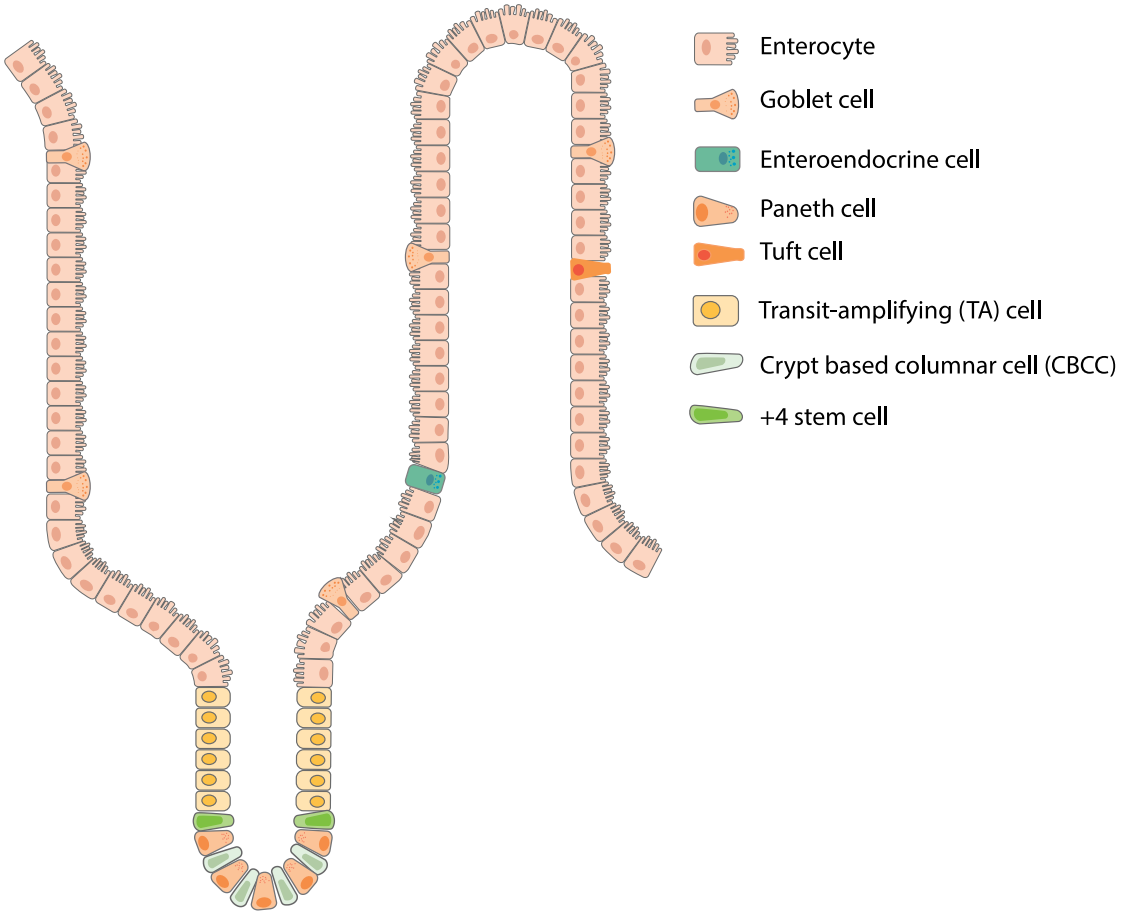

Figure 2-1. Small intestinal epithelial cell organization in mammals. Figure drawn by M. Jia, adapted from Carulli et al. (2014).

Intestinal stem cells and signaling

In the crypt of mammals, there are two populations of intestinal stem cells, including crypt based columnar cells (CBCC) and +4 stem cells. CBCC are active stem cells scattered between Paneth cells. CBCC are responsible for crypt maintenance by proliferation and for epithelial cell turn-over by partially differentiating into proliferating TA cells (Carulli et al., 2014). The +4 stem

cells are named by their position in the bottom of crypts. They are a quiescent population of stem cells with a slow cycling rate and play an essential role in intestinal stem cells regeneration during injury (Carulli et al., 2014).

Chicken intestinal stem cells are also restricted to the small intestinal crypt. The stem cells were identified by in situ hybridization using probes for the marker genes, leucine-enriched G protein-coupled receptor 5 (Lgr5) and olfactomedin 4 (Olfm4) (Zhang and Wong, 2018). Only few studies investigated the molecular mechanism of chicken intestinal stem cell renewal and differentiation. In mammals, the proliferation and differentiation of intestinal stem cells are mainly involved in Wnt/ β catenin signaling and Notch signaling from Paneth cells and mesenchymal cells (Yan et al., 2017; Zhang et al., 2019b). In canonical Wnt/ β catenin signaling pathway, Wnt proteins bind to its receptor, Frizzled protein, and its coreceptor, which results in the dissociation of a destruction complex. These results in the accumulation of β catenin, which then translocates into the nucleus and binds to the transcription factor TCF/LEF family to activate expression of target genes such as Atoh 1 that drives differentiation towards the secretory linage (Yang et al., 2001; Durand et al., 2012; Zhang et al., 2019b). Lgr5 is a widely used marker gene for intestinal stem cells and amplifies canonical Wnt signaling by binding to its ligand R-spondins and the co-receptor (Barker et al., 2007; Hao et al., 2012; Steinhart and Angers, 2018). Yan et al. (2017) reported that only Wnt protein cannot induce intestinal stem cell self-renewal. Crypt intestinal stem cell proliferation and self-renewal can only be activated in the presence of both Wnt protein and R-spondin ligands.

Notch signaling also plays an important role in intestinal stem cell homeostasis (VanDussen et al., 2012). Notch ligands Dll1/Dll4 binds to Notch receptors Notch1/2, which induce the cleavage of extracellular domain and intracellular domain of Notch receptors. The

Notch intracellular domain (NICD) enters into the nucleus and interacts with the transcriptional activator complex to promote expression of target genes, like Hes1 (Demitrack and Samuelson, 2016). Hes1 is an inhibitor of Atoh1, thus, Notch signaling can regulate the fate of progenitor cells into absorptive cells or secretory cells by altering Atoh1 expression. Notch signaling also functions to induce proliferation of intestinal stem cells and progenitor cells (Fre et al., 2005). Olfm4 is another target gene of Notch signaling that encodes a glycoprotein of the olfactomedin family, and functions as a regulator of intestinal inflammation and cell proliferation (VanDussen et al., 2012; Liu et al., 2016). Olfm4 has been widely used as a robust marker for intestinal stem cells due to its high expression and specificity in $Lgr5^{+}$ stem cells (Van der Flier et al., 2009a; b).

Transit-amplifying (TA) cells

Rapid cycling TA progenitors undergo only a limited number of cell divisions before becoming differentiated (Umar, 2010). Absorptive progenitors differentiate into mature absorptive enterocytes, and secretory progenitors differentiate into mature secretory cells, such as Paneth, goblet, enteroendocrine, and tuft cells. Both types of TA cells are highly proliferative, while the proliferative rate of absorptive progenitors are 3.8 fold greater than that of secretory progenitors, which may explain the difference of mature cell numbers (Sanman et al., 2021).

Proliferating cells undergo four phases of the cell cycle, including the G1 (cell growth, cell size increase), S (DNA synthesis), G2 (further cell growth, and preparation for mitosis), and M (mitosis) phases. PCNA (proliferating cell nuclear antigen) protein has been widely used as a proliferation marker due to its increasing expression in the G1 and S phases of the cell cycle and participation in DNA replication. However, nuclear Ki67 is more sensitive and specific for identification of proliferating cells, because it is only expressed in cycling cells that are in the active phases of the cell cycle (G1, S, G2, and M) and not in resting cells in the G0 phase

(Montgomery and Breault, 2008; Bologna-Molina et al., 2013; Juríková et al., 2016). Thus, the proliferation marker Ki67 is also expressed by cycling $Lgr5^+$ CBCC, while Lgr5 and Olfm4 are only expressed by CBCC, not TA cells (Umar, 2010). Proliferating cells in mammals were distributed in the crypt, while the proliferating zone in chickens is located in both the crypt and villus regions (Uni et al., 1998). At day of hatch, most epithelial cells were proliferative, however, the proportion of proliferative cells in the villus and crypt decreased with age during the first week (Geyra et al., 2001).

Intestinal epithelial cell proliferation and differentiation are affected by many factors. In young chicks, *Lactobacillus reuteri* supplementation increased the number of $PCNA^+$ proliferating cells in the jejunum by activation of Wnt/ β catenin signaling, and increased the number of goblet cells stained by periodic acid-Schiff in the jejunum by inhibition of Notch signaling (Xie et al., 2019). Feeding upon hatch increased crypt cell proliferation and the number of differentiated cells including enterocytes and goblet cells of young chicks compared to delayed feeding after 24 hours post hatch (Reicher et al., 2020).

Enterocytes

Enterocytes are also called absorptive cells, which account for the greatest proportion (more than 80%) of small intestinal epithelial cells (Zhang et al., 2019b). The microvilli structure in the apical surface of enterocytes forms a brush-border membrane and increases the surface area for nutrient digestion and absorption (Ferrer et al., 1995; Reicher and Uni, 2021). Proteomic analysis of the chicken small intestine revealed that digestive enzyme and nutrient transporters are located in the brush-border membrane of small intestinal epithelia (Gilbert et al., 2010). These enzymes and transporters support nutrient uptake from the gut lumen to the enterocytes. These

nutrients are either utilized for intestinal maintenance or are transported into the blood circulation by transporters in the basolateral membrane (Kong et al., 2018).

Carbohydrate digestive enzymes and monosaccharides transporters are located in the small intestine of chickens. Paris and Wong (2013) showed that sucrase isomaltase (SI), which breaks down sucrose and isomaltose, was expressed in the jejunum. Sodium glucose transporter 1 (SGT1) and glucose transporters (GLUT2 and GLUT5) are also located in the small intestine to transport glucose into enterocytes or across the basolateral membrane (Paris and Wong, 2013; Miska and Fetterer, 2017). These findings confirm the digestive and absorptive functions of the small intestine for carbohydrate metabolism.

Aminopeptidase N (APN), which converts oligopeptides from the gut lumen into amino acids, is also expressed in the small intestine. These amino acids are then transported into enterocytes by amino acid transporters (Olsen et al., 1997; Paris and Wong, 2013; Miska and Fetterer, 2017). Amino acid transporters including EAAT3 (excitatory amino acid transporter 3), rBAT, B^oAT, and $b^{o,+}AT$ are also expressed in the brush border membrane of the small intestine and transport specific amino acids into enterocytes (Paris and Wong, 2013; Miska and Fetterer, 2017). Amino acid transporters located on the basolateral membrane of enterocytes such as CAT1, CAT2, y^+ LAT1, and y^+ LAT2, transport specific amino acids from enterocytes to blood vessels (Paris and Wong, 2013; Miska and Fetterer, 2017).

The oligopeptide transporter PepT1 is mainly expressed in the duodenum of the small intestine of chickens to transport di- and tri-peptides across the brush-border membrane of enterocytes (Gilbert et al., 2007). PepT1 expression was affected by diet and *Eimeria* infection. Diets with higher levels of protein increased PepT1 expression in all three segments of the small intestine (Chen et al., 2005). *Eimeria acervulina* and *E. maxima* decreased PepT1 expression in

the duodenum and ileum, respectively, while *E. maxima* did not affect PepT1 expression in the jejunum (Paris and Wong, 2013; Miska and Fetterer, 2017). Due to its exclusive expression in enterocytes, PepT1 can be used as a marker gene for intestinal enterocytes in chickens (Zhang and Wong, 2017; Liu et al., 2020).

Goblet cells

Goblet cell are important epithelial cells along the intestinal villi, because they secrete mucin. Mucin is a glycoprotein, which forms a mucus layer that acts as a physical-chemical barrier against pathogens (Liu et al., 2020). Depending upon the structure, mucins can be either neutral or acidic. Goblet cells containing neutral mucins can be detected by periodic acid-Schiff (PAS) staining, while goblet cells that secrete acid mucin can be stained by alcian-blue (AB) (Smirnov et al., 2006). Mucin 2 (Muc2) is a member of the mucin family and is the primary component of the mucus layer. In addition to mucin staining, goblet cells along the intestinal villi have also been identified by in situ hybridization with a Muc2 probe. The density of $Muc2^+$ cells in the villi was greater than that of cells expressing mucin glycoprotein (Reynolds et al., 2020). There were also $Muc2^+$ cells in the crypt, which is also the location of chicken intestinal stem cells expressing Olfm4. This result suggests that the $Muc2^+$ cells in the crypt are pre-goblet cells that might be differentiated from stem cells but have not yet migrated out of the crypt (Liu et al., 2020). Alternatively these $Muc2^+$ cells in the crypt might be multi-functional cells, with the ability to secrete mucin and be able to generate mature intestinal cells (Reynolds et al., 2020).

Mucin mRNA expression and the number of goblet cells are affected by diet, such as dietary essential oils. Zeinali et al. (2017) reported that essential oil increased Muc2 mRNA expression, while decreasing the number of goblet cells. In-ovo feeding of carbohydrates increased both mucin mRNA expression and goblet cells expressing acidic mucin (Smirnov et al., 2006).

Delayed access to feed decreased Muc2 mRNA expression and altered goblet cell distribution in the villi (Liu et al., 2020).

Enteroendocrine cells

Enteroendocrine cells account for a limited proportion (around 1%) of the intestinal epithelial cell population, but play an important role in the enteric endocrine system by secretion of regulatory peptides (Sternini et al., 2008). Enteroendocrine cells were reported to regulate nutrient digestion by alteration of peptide hormone secretion and modulate intestinal inflammation by releasing cytokines (Worthington et al., 2018). Food ingestion and gut microbiota stimulate the secretion and release of hormones from enteroendocrine cells, via several signaling pathways (Gribble and Reimann, 2019). Traditionally, enteroendocrine cells were identified based on the secretion of individual hormones (Worthington et al., 2018). However, recent studies showed that hormone peptides colocalized in the same enteroendocrine cells (Fothergill et al., 2017). Enteroendocrine cells in the chicken act as sensor of nutrients in the gut and subsequently regulate feed intake. However, the interaction between enteroendocrine cells and the microbiota in the gut of chickens still need to be further investigated.

Paneth cells

Paneth cells are differentiated from secretory progenitors. Unlike other mature secretory cells that move up towards the tip of the villi and turnover rapidly, Paneth cells in mammals move down into the bottom of the intestinal crypt (crypts of the Lieberkühn) with a long turn-over time around 18-23 days (Porter et al., 2002). Paneth cells play an essential role in the cellular host defense system. They contain acidophilic granules, which would release many types of microbiocidal agent such as lysozyme, and antimicrobial peptides including α- defensins into the intestinal lumen that act against bacteria, fungi, and viruses (Lueschow and McElroy, 2020). In

addition, Paneth cells can also support $Lgr5^+$ stem cell renewal and maintenance in vivo or in vitro by expression of ligands of Wnt signaling and Notch signaling, such as Wnt3 and Dll4 (Sato et al., 2011).

Paneth cells are also found in the small intestine of chickens. However, their exact location is still controversial. Nile et al. (2004) showed that lysozyme was expressed along the villi but not in the crypt in the small intestine of chickens. This study used immunohistochemistry with an antibody against chicken egg white lysozyme. However, Wang et al. (2016b) reported the presence of Paneth cells in the small intestinal crypt of 6 month old chickens by a combination of histological staining with phloxine-tartrazine and in situ hybridization for lysozyme c (Lyz c). They localized Paneth cells in the crypt of the small intestine. In contrast, the identification of stem cells using Olfm4 in our lab showed that $Olfm4^+$ intestinal stem cells were distributed throughout the entire crypt of chicken intestine, with no $Olfm4^-$ Paneth cells (Zhang and Wong, 2018). Thus, the identification and distribution of Paneth cells still needs to be investigated.

Expression of host defense peptides in the small intestine of chickens

The composition and function of host defense peptides (HDP) in chickens were introduced in the section on embryonic development of the YS. β defensins, cathelicidins (CATH), and LEAP2 are all expressed in the small intestine of broiler chickens. These proteins play important roles in the innate immune system via their broad activity in killing pathogens. The expression of avian HDPs showed an age-dependent pattern. Compared to DOH, AvBD1 and AvBD2 decreased in the duodenum and ileum during the first week post-hatch, but increased in the duodenum in the second week post-hatch (Bar-Shira and Friedman, 2006). CATH2 was expressed in heterophils exclusively, and the number of CATH2 stained heterophils was relatively greater in the small intestine of neonatal chicks than older chicks (Cuperus et al., 2016). Interestingly, this study

revealed that AvBD9 was expressed by enteroendocrine cells in the small intestine, which co-expressed the enteroendocrine product serotonin. LEAP2 was identified by in situ hybridization in the epithelial cells along the villi but not in the tip of the villi or the crypts (Su et al., 2017). Lynn et al. (2004) reported that LEAP2 was highly expressed in the small intestine of chickens. The intensity of the LEAP2 PCR product was much greater than the AvBDs in the small intestine, which suggested that LEAP2 may play an important role in the enteric defense system in the small intestine.

LEAP2 mRNA abundance was consistently downregulated by *Eimeria* infection in the small intestine (Casterlow et al., 2011; Paris and Wong, 2013; Su et al., 2017). LEAP2 mRNA abundance in the jejunum was also decreased by co-infection with *Eimeria spp.* and *C. perfringens.* This repression of LEAP2 mRNA, was reversed by supplementation with microencapsulated sodium butyrate (Song et al., 2017). Oral infection with *Salmonella* induced the expression of LEAP2 in the small intestine in vivo. Townes et al., (2004) had previously shown that LEAP2 showed antimicrobial activity against *Salmonella spp.* in vitro (Townes et al., 2004). However, Garcia et al. (2020) reported that *Salmonella* Typhimurium challenged chickens had a lower expression level of LEAP2 in the ileum at 5 day post infection. Thus, LEAP2 expression can be modulated by the microbiota and parasites, which subsequently affects the innate defense. In ovo feeding of probiotics increased LEAP2 expression in the ileum of necrotic enteritis challenged chickens at D7 (White, 2021), which suggests that probiotics may promote LEAP2 expression in pathogen-challenged chickens.

Tight junctions in the small intestine

The intestinal epithelia are not only the site for nutrient absorption, but also serves as a primary component of the physical barrier that prevents pathogens from crossing the intestinal

epithelium (Lee et al., 2018). The intercellular junctions contact the plasma membrane of adjacent cells and create a mucosal barrier, which mainly consists of tight junctions, gap junctions, adherens junctions, and desmosomes (Shini et al., 2021) (Figure 2-2). Tight junctions seal the apical plasma membrane of adjacent cells to regulate paracellular transport of ions and water. The adherens junction together with the tight junction form the apical junctional complex, which is the primary structure of the intestinal barrier (Shini et al., 2021). The gap junction supports small molecule transportation by channel formation (Rübsam et al., 2018; Bhat et al., 2019). Similar to the adherens junction, desmosomes function for cell-cell adhesion, but does not interact with the cytoskeleton (Rübsam et al., 2018; Shini et al., 2021).

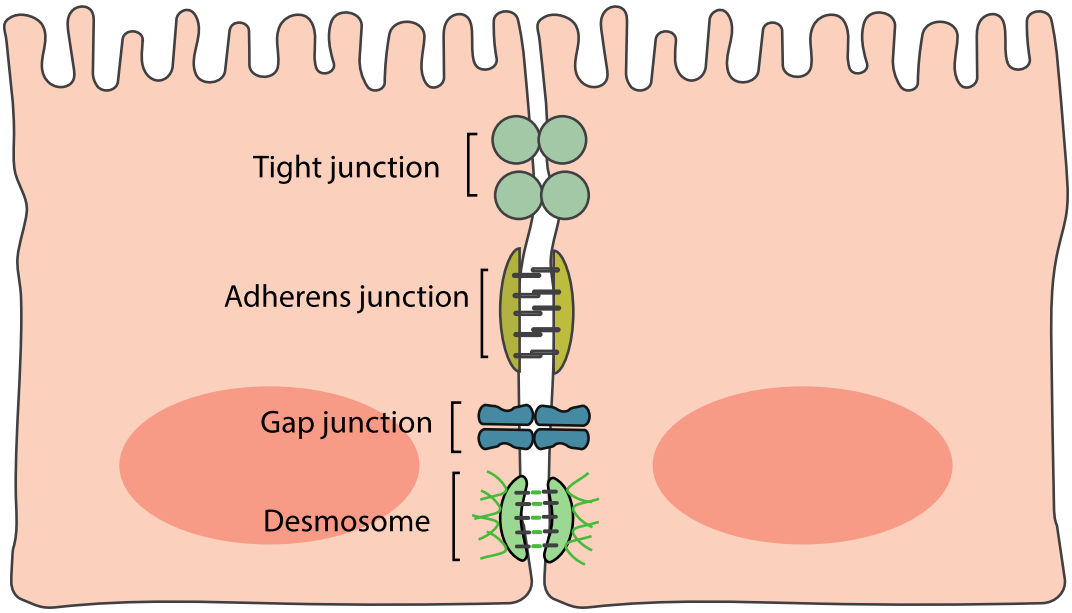

Figure 2-2. The types of intercellular junctions contacting the plasma membrane of adjacent epithelial cells. Figure drawn by M. Jia, adapted from Kutova et al.(2020).

The composition and function of tight junctions have been widely investigated recently. Tight junctions are a complex consisting of transmembrane proteins and scaffolding proteins like zonula occludens (ZO). ZO provides the structure for recruitment of proteins to the cytoplasmic surface of junctions (Figure 2-3). ZO1, ZO2 and ZO3 connect the transmembrane tight junction to

the actin cytoskeleton. This interaction can regulate cytoskeleton reorganization (Ulluwishewa et al., 2011). Claudins (CLDN) are considered as the backbone of tight junctions and are tetra-span transmembrane proteins with two extracellular loops. There are 23 members in the CLDN family. CLDN1 tightens the tight junction and decreases paracellular permeability, while CLDN2 loosens tight junctions by formation of pores (Ulluwishewa et al., 2011). Inoculation of young broilers with a ceca fermentation product improved intestinal integrity by increasing CLDN1 and decreasing CLDN2 expression (Gong et al., 2020). Occludin (OCLN) is also a tetra-span protein that is another main component of tight junctions. The location of OCLN can shift into the cytoplasm during exposure to stimuli such as inflammation, resulting in an increase in intestinal epithelial permeability (Von Buchholz et al., 2021). Junctional adhesion molecules (JAM) are single transmembrane tight junction proteins that regulate tight junction protein assembly (Luissint et al., 2014). JAM A (JAMA) regulates intestinal barrier function by altering expression of CLDNs (Garcia-Hernandez et al., 2017). Interestingly, JAMA knock-out mice had a higher level of proliferating intestinal epithelial cells, which suggests that JAMA restricts intestinal epithelial cell proliferation (Nava et al., 2011). JAM2 plays an important role in intestinal barrier function. The expression level of JAM2 in the duodenum of *E. acervulina* challenged chickens was increased by a specific strain of *Bacillus subtilis* (Wickramasuriya et al., 2021).

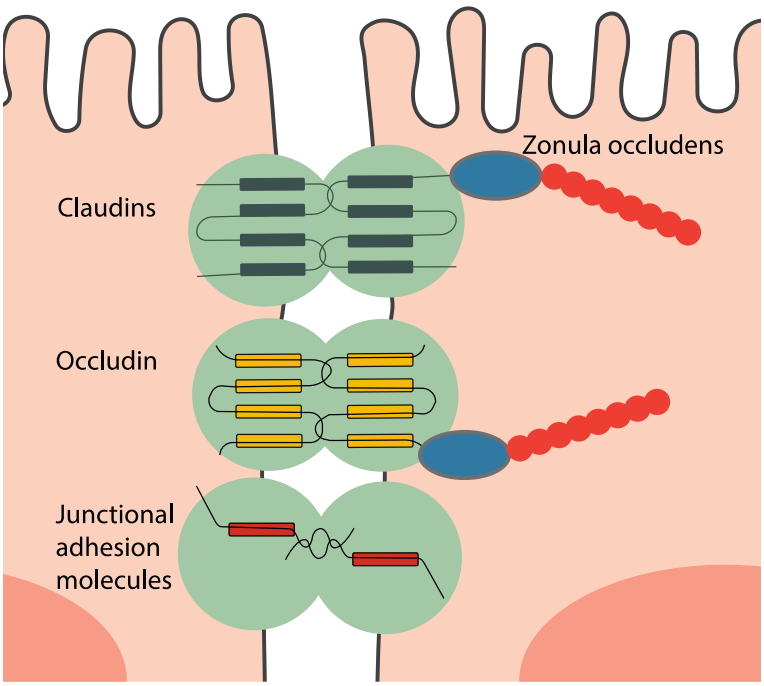

Figure 2-3. The composition of tight junctions. Figure drawn by M. Jia, adapted from Collins et al. (2017).

Summary

Both the YS and small intestine are derived from embryonic splanchnopleure. Thus, they share similar structure and functions. The YS and small intestine have similar villi-like structures to increase the surface area for nutrient digestion and absorption and are lined with various types of epithelial cells. Tight junctions seal adjacent cells to maintain their structural integrity. The YS and small intestine are both multi-functional organs. They can not only uptake nutrients (lipids, carbohydrates, proteins, amino acids, minerals, etc.) but also secrete antimicrobial peptides to enhance immunity. However, their functions can be affected by environmental effectors, such as heat stress or pathogens. Heat stress during incubation affects yolk utilization and gene expression involved in nutrient metabolism, which might be related to the high mortality and low hatchability of chicken embryos. Pathogens impair intestinal morphology and integrity, and affect enteric immunity. Probiotics, however, can alleviate the harmful effects of pathogens to improve chick health.

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Chapter II Objectives and Hypotheses

The small intestine and yolk sac (YS) share a similar structure of villi and functions of nutrient digestion and absorption during the embryonic or post-hatch stages in chickens. Recent studies showed that young chicks were susceptible to pathogens during the first week, and probiotics improved chicken gut health and intestinal morphology in both healthy and challenged chicks. However, there were few studies to show the effects of probiotics on small intestinal epithelial cell proliferation and differentiation, antimicrobial peptide expression, and tight junction expression in healthy chickens. High incubation temperature was reported to decrease hatchability of chickens, while there was no research regarding the effect of heat stress on the structure of the YS, which might relate to the low yolk utilization of heat-exposed chick embryos. AvBDs showed a similar expression pattern in the YS of embryonic chickens with a peak expression level in the middle of the incubation period, but their role in the innate defense system in the YS was not clear. Thus, to answer these questions, my objectives and hypotheses were as follows.

Objectives:

1. To investigate the effect of probiotics on ontogeny of small intestinal epithelial cells, including stem cells, proliferating cells, enterocytes, goblet cells, and mRNA abundance of antimicrobial peptides in young chicks.

2. To localize tight junction protein expression in intestinal epithelial cells using in situ hybridization, and determine the effect of probiotics on expression of tight junction proteins in the small intestine of young chicks.

3. To profile the expression of tight junction proteins in the YS of embryonic broilers, and to detect the effect of high incubation temperature on tight junction protein expression in the YS of embryonic chicks.

4. To compare the expression of avian host defense peptides AvBD10 and CATHs in the YS of embryonic broilers and layers.

Hypotheses:

1. Probiotics would lengthen intestinal villi by increasing epithelial cell proliferation and differentiation in broiler chicks.

2. Probiotics would improve intestinal integrity by increasing expression of tight junction proteins that tighten the tight junction.

3. Tight junction proteins would be expressed in the YS, and their expression could be affected by high incubation temperature.

4. The expression of avian host defense peptides would show a similar pattern in the YS of broilers and layers.

Chapter III Effect of probiotics on the morphology of intestinal villi and the ontogeny of intestinal epithelial cells in young chickens

Abstract

Probiotics consist of beneficial bacteria that enhance intestinal morphology and function. However, the mechanisms responsible for this effect have not been determined. The objective of this study was to determine the effect of probiotics on intestinal villus morphology and ontogeny of intestinal epithelial cells in broiler chickens and further to investigate the relationship between them. Day of hatch Cobb 500 chicks were randomly divided into 3 groups: Basal diet (Control), basal diet + Probiotic 1, and basal diet + Probiotic 2. Each treatment group contained 6 replicate cages with 11 chicks/cage. At days 2, 4, 6 and 8, all chicks were weighed and one chick from each replicate cage was randomly selected and used for collection of duodenum, jejunum, and ileum (n = 6). The RNAscope in situ hybridization procedure was used with probes for olfactomedin 4 (Olfm4) to identify stem cells and define the intestinal crypt. The crypt depth (CD) and villus height (VH) were measured. The mRNA abundance of a marker gene for absorptive cells (Peptide transporter 1, PepT1), proliferating cells (Ki67), goblet cells (Mucin 2, Muc2), stem cells (Olfactomedin 4, Olfm4), and an antimicrobial peptide (liver-enriched antimicrobial peptide 2, LEAP2) were examined by qPCR. The body weight data were analyzed by one-way ANOVA using JMP Pro15. The intestinal weight and morphology were analyzed by two-way ANOVA and Tukey's test with two main factors including treatment and age. The mRNA expression of marker genes was analyzed by three factorial analysis (treatment, age, and sex) and Tukey's test. The significance level was set at 0.05. Probiotic 1 and Probiotic 2 decreased body weight gain from day 2 to day 4 but did not significantly affect body weight of newly hatched chicks. Probiotic supplementation

did not improve intestinal weight and morphology. However, Probiotic 1 and 2 transiently increased marker gene expression for proliferating cells (Ki67) and decreased LEAP2 mRNA abundance in the jejunum. Probiotic 1 increased Ki67 mRNA in the ileum, but decreased PepT1 mRNA abundance in the duodenum. These results show that probiotic supplementation to newly hatched chicks for one week did not improve growth performance and intestinal morphology, but may improve intestinal epithelial cell proliferation, and reduce host enteric defense from LEAP2 antimicrobial peptide. Probiotic 1 may decrease peptide uptake in the duodenum compared with the Control.

Keywords: probiotics, proliferation, epithelium, intestine, in situ hybridization

Introduction

Probiotics, also called direct fed microbials (DFM), consist of a variety of beneficial bacteria, such as *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Bacillus* that are tolerant to acid and bile (Fuller, 1989; Hyronimus et al., 2000; Pennacchia et al., 2004). Probiotics have been used as a live microbial supplement to enhance animal performance by competitively excluding pathogenic microorganisms, modulating the immune system and improving the epithelial barrier via adhesion to the cell monolayer (Bermudez-Brito et al., 2012). Increased body weight and improved feed conversion efficiency have been reported in broiler chickens after feeding probiotic-supplemented diets (Patel et al., 2015). A morphological change associated with probiotic supplementation is an increase in villus height, which contributes to an increase in absorptive surface area and enhancement of nutrient uptake (Amat et al., 1996; Awad et al., 2008). However, the mechanism of the interaction between probiotics and ontogeny of intestinal epithelial cells in chickens is still unclear.

Crypt base columnar cells (CBCC) are a type of active intestinal stem cells that are located in the crypt. CBCC are able to self-renew and play an important role in intestinal epithelial cell homeostasis via differentiation into mature epithelial cells (Umar, 2010). The Olfm4 gene is a robust marker of intestinal stem cells due to a high expression level (Van der Flier et al., 2009; Zhang and Wong, 2018). Ki67 protein has been widely used as a proliferation marker that is highly expressed in cycling cells (G1, S and G2 phases) but not in resting cells (G0 phase) (Muskhelishvili et al., 2003). Thus, Ki67 mRNA has also been used as a marker for proliferating cells. The enterocytes are absorptive cells that migrate up the villi and express a variety of transporters responsible for the uptake of nutrients, such as amino acids, peptides, carbohydrates and minerals (Smith, 1985; McKie et al., 2000; Gilbert et al., 2008). PepT1 is one of the family of proton-coupled oligopeptide transporters that is mainly involved in the transport of dipeptides and tripeptides. PepT1 is a high capacity, low affinity transporter that is located at the apical membranes of intestinal epithelial cells and serves as a marker of absorptive cells (Gilbert et al., 2008; Spanier, 2014; Zhang and Wong, 2017). Goblet cells secrete mucins, especially mucin 2 (Muc2) which provides a physical barrier between the gut contents and cells that line the villi (Reynolds et al., 2020). Therefore, the objective of this study was to determine the effect of dietary probiotics on villus height and mRNA abundance of Olfm4, Ki67, PepT1, and Muc2 to determine the number and distribution of small intestinal stem cells, proliferating cells, enterocytes, and goblet cells in broiler chickens, respectively. LEAP2 mRNA expression was also analyzed to assess the effect of probiotics on an antimicrobial peptide expressed by intestinal epithelial cells.

Materials and Methods

Animals and tissue collection

Cobb 500 broiler eggs were obtained from a local hatchery, and transferred to the Virginia Tech poultry farm for incubation at 37.5 °C. On day of hatch (doh), 6 of 204 chicks were euthanized by cervical dislocation for sample collection. Three segments of small intestine (duodenum, jejunum and ileum) were collected, the contents were removed, and the tissue was rinsed with cold 1 × PBS (Phosphate buffered saline) and weighed. A piece from the middle of each segment was minced and rapidly frozen on dry ice, then stored at -80 °C. Around 3 cm of each segment were fixed in 10% buffered formalin for 24 h, then transferred into 70% ethanol prior to paraffin embedding (StageBio, Mount Jackson, VA). The remaining 198 chicks were weighed, wing-banded, and randomly divided into 3 dietary treatment groups: basal diet without probiotic supplement (Control), basal diet + 0.5 g/kg PoultryStar® probiotic-based synbiotic product (Probiotic 1), and basal diet + 1.5 g/kg PrimaLac (Probiotic 2). The dosages were according to the recommendation from the companies. Each group had 6 replicate cages with 11 chicks per cage. The chicks received ad libitum access to water and feed. At day (d) 2, d4, d6, and d8, all chicks were weighed. One chick from each cage was euthanized and intestinal samples (duodenum, jejunum, and ileum) were collected and weighed as described above at d2, d4, and d6, but not at d8. This is because only the effect of probiotics on intestinal morphology and marker gene expression of intestinal epithelial cells during the first week were considered in this study. Intestinal epithelial cell proliferation and differentiation are more active in young chicks, which might be more readily affected by environmental factors. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Sex determination

The sex of chicks collected on d2, d4 and d6 were determined by PCR. Genomic DNA was isolated from frozen duodenal tissues (~25mg) using the Quick-gDNA™ MiniPrep Kit (Zymo Research, Irvine, CA). DNA was quantified by Nanodrop and diluted to 10 ng/ µl to be used for chicken sexing. PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler using the following conditions: 95 °C for 10 mins; 32 cycles of 95 °C for 30 s; 55 °C for 30 s; 72 °C for 2 mins. Each PCR reaction contained 1 µl forward and 1 µl reverse primers (5 µM for the W chromosome (Forward: 5'-CTGTGATAGAGACCGCTGTGC-3' and Reverse: 5'-CAACGCTGACACTTCCGATGT-3'), 1 µl forward and reverse primers (5 µM for the autosomal tyrosinase gene (Forward: 5'-TCGAGAGGCATAATAATGCATCCA-3' and Reverse: 5'-AGAGCTTGCTGAGGAAGGAGTG-3'), 1 µl genomic DNA (10 ng/ μl), 7.5 µl DEPC water, and 12.5 µl AmpliTaq Gold 360 Master mix (Thermo Fisher Scientific) (Gilbert et al., 2007; Kaminski and Wong, 2018). The PCR products were separated on 1% agarose gels. Male chicks (ZZ) exhibit only 1 band at 400 bp (tyrosinase). Female chicks (ZW) exhibit 2 bands at 400 bp (tyrosinase) and 1200bp (W chromosome). The sex of male chicks was verified by repeating the PCR-agarose gel procedure.

RNA extraction, cDNA synthesis, and real time qPCR

Frozen tissue (25 - 35 mg, n = 6) were homogenized in TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) using a TissueLyser II (Qiagen, Hilden, Germany). RNA was extracted using the Direct-zol RNA Miniprep Plus Kits (Zymo Research, Irvine, CA). One µg of total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The quantitative PCR (qPCR) procedure was conducted in an Applied Biosystems 7500 Fast Real-time PCR system (Thermo

Fisher Scientific) with the fast program: 95 °C for 20 s, 40 cycles of 90 °C for 3 s followed by 60 °C for 30 s. The PCR reaction consisted of 5 µl Fast SYBR Green Master Mix (Thermo Fisher Scientific), 1 µl of each forward and reverse primers (5 µM), 1.5 µl DEPC water, and 1.5 µl diluted cDNA (1:20). Each sample was run in duplicate. All primers for qPCR are listed in Table 3-1. Ribosomal proteins (RPLP1 and RPL4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and TATA box binding protein associated factors (TAF2 and TAF4) were tested as reference genes. GeNorm analysis was performed in qbase+ to identify the three most stable reference genes, which were RPL4, GAPDH, and TAF4. The geometric mean of the Ct values for these three reference genes was used to calculate the ΔCt value of target genes. The average ΔCt in the Control group at d2 was used as the calibrator. The fold change of gene expression was analyzed using $2^{-\Delta\Delta Ct}$.

In situ hybridization analysis

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into tissue sections (5 - 6 µm) with a microtome (Microm HM 355S; Thermo Fisher Scientific) and placed on Superfrost-Plus glass slides (Electron Microscopy Sciences, Hatfield, PA). Tissue sections were baked at 60 °C for 1 h, deparaffinized with xylene, and rinsed with 100% EtOH. The sections were then incubated with hydrogen peroxide to block peroxidase activity, boiled in target retrieval solution, and treated with protease. Probes for in situ hybridization were designed by Advanced Cell Diagnostics (ACD, Newark, CA). Probes for Olfm4, Ki67, PepT1, Muc2 and LEAP2 were used to identify intestinal stem cells, proliferating cells, enterocytes, goblet cells, and cells expressing antimicrobial peptides, respectively. The RNAscope in situ hybridization assay was performed using the RNAscope™ 2.5 HD Assay-BROWN or RED kit (ACD) according to the manufacturer's instructions. Sections were counterstained with 50% hematoxylin solution Gill II

(Sigma-Aldrich, St. Louis, MO). The images were captured using a Nikon Eclipse 80i microscope and a Nikon DS-Ri1 camera.

Intestinal morphological measurement

The Olfm4 stained images (n = 5) were used to measure villus height (VH) and crypt depth (CD). Crypt depth was defined as the depth of the Olfm4 stained region, and VH was measured from the villus tip to the top of the crypt stained by Olfm4 (Liu et al., 2020). The villus height/crypt depth (VH/CD) ratio was calculated.

Statistical analysis

Data were analyzed using JMP Pro 15 (SAS Institute Inc., Cary, NC). For body weight analysis, cage was considered as the experimental unit. One-way ANOVA and Tukey's test were used to compare the body weight difference between treatments within day from doh to d8. For intestinal weight, morphology, and gene expression, chick was considered as the experimental unit. Data were analyzed by two-way ANOVA and Tukey's test from d2 to d6 for intestinal weight and the morphology data. Since the fold change of gene expression did not show a normal distribution, the data were first normalized by log10 transformation, which was the single transformation method that best fit all of the tested genes. The transformed data were analyzed using three factorial analysis and Tukey's test with three main effects including treatment, age, and sex. Significant difference was set as $P < 0.05$.

Results

Body weight and intestinal weight

The BW and BWG for the 3 treatments are shown in Table 3-2. Probiotics did not significantly affect BW from d2 to d8. There was a significant decrease of BWG from d2 to d4 (*P*

< 0.05) for both Probiotic 1 and Probiotic 2 compared to Control, that resulted in trends for decreases in BW at d4 ($P = 0.052$) and d6 ($P = 0.09$).

Absolute intestinal weight and relative intestinal weight (absolute intestinal weight / body weight) for the duodenum, jejunum, and ileum are shown in Figure 3-1. The absolute and relative intestinal weights for duodenum, jejunum and ileum were not affected by probiotics at d2, d4, and d6 ($P > 0.05$). However, absolute intestinal weight and relative intestinal weight were altered by age. For all three dietary treatments, the absolute weight of duodenum, jejunum, and ileum was significantly increased from d2 to d4 and d4 to d6. Relative intestinal weight increased in the duodenum from d2 to d4 for all treatments. The jejunal relative weight was significantly increased in the Control and by Probiotic 2 from d2 to d4, but not by Probiotic 1. In the ileum, the relative intestinal weight was increased from d2 to d6 by Probiotic 1 and 2, which was not observed in the Control.

Intestinal Morphology

Intestinal VH was not affected by probiotics; however, it was affected with age in the duodenum, jejunum, and ileum. VH in the duodenum and ileum increased from d2 to d4 and from d4 to d6, while in the jejunum there was an increase in VH from d2 to d4 (Table 3-3).

The CD was affected by treatment in the ileum. Probiotic 2 decreased the CD in the ileum compared with the Control and Probiotic 1 (Table 3-3). The CD was also altered with age in all three intestinal segments (Table 3-3). There was an increase in CD from d2 to d4 in the duodenum and jejunum, while in the ileum there was an increase in CD from d2 to d4 and a decrease from d4 to d6.

For the VH/CD ratio, there was an interaction between treatment and age in the ileum (Table 3-3). The VH/CD ratio in the ileum in the Control and Probiotic 2 did not change from d2

to d4 and then increased from d4 to d6. However, Probiotic 1 increased the VH/CD ratio from d2 to D4, with no change from d4 to d6. (Figure 3-2). The VH/CD ratio increased from d4 to d6 in the duodenum and from d4 to d6 in the jejunum (Table 3-3).

Relative expression of marker genes

In the duodenum, there was a treatment × age interaction for Ki67 and LEAP2 mRNA abundance (Table 3-4). Ki67 mRNA was decreased by Probiotic 1 from d2 to d4 followed by an increase from d4 to d6 (Figure 3-3). There was no change in Ki67 mRNA in the Control and Probiotic 2 from d2 to d6. LEAP2 mRNA did not change between d2 to d6 in the Control, but both Probiotic 1 and Probiotic 2 increased from d4 to d6 (Figure 3-4). There was also an age × sex interaction for LEAP2 in the duodenum (Table 3-4). LEAP2 was increased from d4 to d6 for males, but not changed from d2 to d6 for females (Figure 3-5). There were main effects of treatment and sex only for PepT1 in the duodenum (Table 3-4). Probiotic 1 decreased PepT1 mRNA abundance in the duodenum compared with the Control. Males had greater PepT1 mRNA abundance in duodenum than females. There was a main effect of age for Olfm4 and Muc2 mRNA in the duodenum (Table 3-4). Olfm4 mRNA increased from d2 to d6, while Muc2 mRNA increased from d2 to d4.

In the jejunum, there was an interaction between treatment and age for Ki67 and LEAP2 (Table 3-5). Ki67 mRNA abundance in the Control did not change between d2 and d4 but increased from d4 to d6 (Figure 3-3). Probiotic 2 upregulated Ki67 mRNA abundance from d2 to d4, and was unchanged from d4 to d6, Probiotic 1 did not affect Ki67 mRNA expression from d2 to d6. LEAP2 mRNA abundance in the Control was unchanged between d2 and d4 and then decreased from d4 to d6 (Figure 3-4). Probiotic 1 and 2 downregulated LEAP2 mRNA abundance from d2 to d4 followed by an upregulation from d4 to d6. There was an age effect for Muc2 in the jejunum

(Table 3-5). There was an increase in Muc2 mRNA from d2 to d4 and d6. Males tended to have greater PepT1 mRNA abundance in the jejunum than females ($P = 0.09$).

In the ileum, there were no treatment × age interactions (Table 3-6). There was a main effect of treatment for Ki67 mRNA. Ki67 mRNA abundance was upregulated by Probiotic 1 compared with the Control. There were also main effects of age for Olfm4, Ki67, and Muc2 mRNA abundance (Table 3-6). Olfm4 mRNA abundance increased from d2 to d4 with no further increase from d4 to d6. Ki67 mRNA decreased from d2 to d4 and then increased from d4 to d6. Muc2 mRNA increased from d2 to d4 and then from d4 to d6. There was a trend for an increase in PepT1 mRNA in the ileum of males than females.

Effect of probiotics on intestinal stem and differentiated cells

Intestinal stem, proliferating, absorptive, and secretory cells were identified by in situ hybridization in the duodenum, jejunum and ileum. Stem cells that expressed Olfm4 were located in the intestinal crypt in the duodenum, jejunum and ileum (Figure 3-6, 3-7 and 3-8). Proliferating cells that expressed Ki67 were located in the crypt, blood vessels in the villi, and the muscularis (Figure 3-9, 3-10 and 3-11). Epithelial cells at d4 expressing PepT1 and LEAP2 mRNA were only present along the intestinal villi, whereas Muc2 mRNA expressing cells were located not only along the villi, but also in the crypt (Figure 3-12). The distributions of stem (Olfm4), proliferating (Ki67), secretory (Muc2, LEAP2) and absorptive (PepT1) cells were similar between control and probiotic treatment.

Discussion

In this study, chicks fed diets supplemented with probiotics did not improve BW. This is not surprising because this was only a short 8-day study. Although the effect of probiotics on BW of broilers is strain-specific, dietary probiotics tend to increase BW of broilers in the later stages

after the age of d8. Karaoglu and Durdag (2005) found that two dosages of probiotics (*Saccharomyces cerevisiae*) significantly increased the live BW of male Ross-308 chicks at d14, d21, or d28, but there was no significant effect on BW at d1 and d7. Alkhalf et al. (2010) showed that a monospecies commercial probiotic containing *Pediococcus acidilactici* significantly improved broiler BW starting from two weeks of age to six weeks of age, but there was no effect on BW in the first week. Baldwin et al. (2018) found that administration with three species of *Lactobacillus* (*L. ingluviei, L. agilis and L. reuteri.*) at doh increased BW of broiler chickens at d28, but decreased BW between d2 and d6 post hatch. In our study, Probiotic 1 and 2 decreased BWG from d2 to d4, which resulted in a trend for decrease in BW at d4 ($P = 0.052$) and d6 ($P = 0.09$). This may be because the young chicks needed a period to adapt to the diets supplemented with probiotics.

The absolute and relative intestinal weights were not affected by probiotic treatments between d2 and d6. Awad et al. (2009) reported that broiler chicks fed a synbiotic or probiotic *Lactobacillus* product for 35d had non-significant higher relative small intestine weights. However, 7 day-old male Ross-300 broiler chickens fed with probiotics (Biosof®) had higher relative duodenum, jejunum, and ileum weights at d 42 compared to the control (Shahir et al., 2014). This result may suggest that the growth of small intestinal segments could be improved by long term exposure to probiotics, but not by short-term exposure. Diversity of probiotic strains may also be a contributing factor. There are few previous studies showing the effect of probiotics on small intestine weight during the early stage.

In our study, probiotics did not affect VH and the VH/CD ratio from d2 to d6. In previous longer-term studies, probiotics increased VH or VH/CD. Supplementation of broiler chickens with *Lactobacillus sp.* for 35 d significantly increased VH/CD in the duodenum and ileum by

numerically or significantly increasing VH and/or decreasing CD (Awad et al., 2009). The addition of a probiotic mixture that included *Bacillus* and *Lactobacillus* to 21 d-old Ross 308 chickens for 20 d increased VH of the jejunum (Song et al., 2014). The increased VH would provide more intestinal villi surface area available for nutrient uptake. The increased VH/CD indicated a greater intestinal epithelial cell turn-over rate (Awad et al., 2009). However, there are few studies that investigate the effect of probiotics on intestinal morphology before one week of age. Thus, supplementation of probiotics improves the growth of villi in long-term studies but not short-term studies.

Probiotics increased intestinal stem cell proliferation. Broiler chickens fed diets supplemented with either *Lactobacillus plantarum 16* or *Paenibacillus polymyxa 10* for 21 d had a higher level of cell proliferation in the ileal mucosa compared to the control (Wu et al., 2019a). Oral administration to 3-day-old chickens with *Lactobacillus reuteri* for 7 d promoted intestinal stem cell proliferation via the Wnt/β-catenin pathway (Xie et al., 2019). Here, Probiotic 1 and 2 significantly increased Ki67 mRNA abundance in the jejunum at d4; however, RNAscope images showed no clear difference in Ki67 mRNA expression in the jejunum between the Control and probiotic treatments. This may be because the small fold change (around 1.5 - 2-fold) of Ki67 mRNA abundance by Probiotic 1 or 2 is not detectable by the RNAscope method. RNAscope in situ hybridization is an excellent way to show the distribution of target mRNA but not a good way to quantify small changes in mRNA. Even though there is software available to count the mRNA signal dots in RNAscope images, there still is difficulty quantifying gene expression especially for highly expressed genes. Thus, we only use RNAscope to show the distribution of Ki67 expressing cells, not for quantifying Ki67 mRNA expression.

PepT1 is a peptide transporter for dipeptides and tripeptides and can be used as a marker gene for enterocytes (Leibach and Ganapathy, 1996; Gilbert et al., 2007; Zhang and Wong, 2017). Pavlova et al. (2016) showed that administration of a probiotic mixture containing *Lactobacilli* to Ross 308 broilers for 15 d did not affect PepT1 expression in the duodenum and jejunum. In the present study, qPCR results showed that PepT1 expression was decreased in the duodenum by Probiotic 1 at d 4. Although not quantified, in situ hybridization showed that PepT1 mRNA-expressing enterocytes were similar between treatments at d4. This indicates that probiotics did not affect the number of enterocytes, although it did affect the mRNA abundance of the peptide transporter. There may be a decrease in the number of PepT1 transporters per cell. The lowered gene expression of PepT1 suggests lower capacity to transport di- or tri-peptides from the diet to intestinal epithelial cells. This could result in less nutrients going through enterocytes, which could explain why the body weight gain from d2 to d4 was decreased by probiotic treatment.

Male chickens had greater PepT1 mRNA in the small intestine than females. Full-factorial ANOVA with age, sex and tissue showed that PepT1 gene expression in duodenum, jejunum, and ileum of males was numerically higher than that in females (P = 0.064) (Kaminski and Wong, 2018). In this study, PepT1 mRNA abundance was greater in the duodenum of males than that of females and tended to be greater in the jejunum and ileum. Due to the function of PepT1, the more expression of PepT1 mRNA indicates a higher capacity to transport di- or tri- peptides from the diet to intestinal epithelial cells. This may explain why the body weight of male chickens are greater than that of female chickens. In situ hybridization showed that PepT1 mRNA was expressed in enterocytes located along the villi. Thus, greater PepT1 gene expression may be associated with greater VH and more surface area to absorb the nutrients, which may partly explain why the growth of male chickens is faster than females.

The epithelium of the small intestine has multiple functions. Besides the absorptive function, epithelial cells can also secrete antimicrobial peptides and mucus to protect the epithelium from luminal pathogens. LEAP2 is one of the antimicrobial peptides expressed in the liver and small intestine (Ge et al., 2018). However, LEAP2 expression was different in healthy versus infected chickens. LEAP2 mRNA abundance was upregulated by infection with *Salmonella* Enteritidis and downregulated by infection with *Eimeria maxima* in the small intestine of chickens (Townes et al., 2004; Su et al., 2017). Pavlova et al. (2016; 2017) reported that after feeding a probiotic mixture (*Lactobacillus brevis, L. plantarum* and *L. bulgaricus*) for 15 d, LEAP2 expression in the liver, duodenum, and jejunum was not changed in healthy broiler chickens.

Mucin 2 gene expression can be affected by the bacterial strain and dose of probiotics (Allen et al., 1998). Chicks fed *Bacillus pumilus* or *Bacillus subtilis* had greater Muc2 mRNA expression in the ileum on d14 (Bilal et al., 2021). Supplementation of *Bacillus subtilis* to broiler chickens for 6 weeks increased Muc2 mRNA expression in the small intestine, while lactic acid bacteria did not affect Muc2 expression (Aliakbarpour et al., 2012).

In the present study, Probiotic 1 and Probiotic 2 decreased LEAP2 mRNA abundance in the jejunum at d4. Probiotic 1 additionally decreased LEAP2 mRNA abundance in the duodenum at d4. Muc2 mRNA abundance was not significantly affected by probiotic treatment in this study. Although not quantified, RNAscope in situ hybridization analysis revealed that the density of LEAP2-expressing or Muc2-expressing secretory cells appeared to be the same. This suggests that probiotics did not affect the number of secretory cells, but may affect the level of LEAP2 mRNA for each cell. However, this study only examined mRNA levels. The protein levels of LEAP2 or Muc2 are unknown.

It is interesting that probiotics did not appear to influence the number of stem, proliferating, absorptive, or secretory cells, but affected mRNA expression of marker genes of proliferating and absorptive cells and mRNA expression of an antimicrobial peptide from secretory cells. Most of the changes in mRNA abundance occurred on d4 after probiotic supplementation. The body weight gain from d2 to d4 was lower in the probiotic groups. This indicates that at an early age (d4), young chicks have not yet adapted to the probiotic product. The probiotics promoted expression of an intestinal proliferating cell marker in the jejunum, but decreased the peptide transporter in enterocytes in the duodenum and decreased the antimicrobial peptide expression in secretory cells, even though the change of mRNA expression of marker genes did not appear to change the number of intestinal epithelial cells.

Summary and Conclusion

In summary, short-term dietary administration of two probiotics for 6-8 days did not improve growth performance and small intestinal weight and morphology of newly hatched broilers. However, probiotic supplementation to chicks increased expression of a proliferating cell marker in the jejunum and ileum, which potentially enhances intestinal cell proliferation. Probiotic supplementation to chicks transiently reduced antimicrobial peptide LEAP2 mRNA abundance in the duodenum and jejunum, which might reduce host enteric defense. Supplementation with Probiotic 1 decreased oligopeptide transporter PepT1 mRNA abundance in the duodenum of chicks, which might inhibit peptide uptake. Male chicken had greater PepT1 mRNA in the duodenum than females, which might relate to their faster growth rate.

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Table 3-1. Forward or reverse primers for qPCR.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Amplicon size	Accession No.
Olfm4	TTGCCGGATACCACCTTTCC	TTTCTGCAAGAGCGTTGTGG	72	NM_001040463.1
Ki67	AAAGGGTCCACGCAAACCT	GGCTGAGAGGGCTTCTGAAA	61	XM_422088.5
PepT1	CCCCTGAGGAGGATCACTGTT	CAAAAGAGCAGCAGCAACGA	66	NM_204365.1 (Gilbert et al., 2007)
Muc2	CTGATTGTCACTCACGCCTTAATC	GCCGGCCACCTGCAT	147	JX284122.1 (Liu et al., 2020)
LEAP2	CTCAGCCAGGTGTACTGTGCTT	CGTCATCCGCTTCAGTCTCA	66	NM_001001606.1 (Su et al., 2017)
RPLP1	TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG	62	NM_205322.1 (Liu et al., 2020)
RPL4	TCAAGGCGCCCATTCG	TGCGCAGGTTGGTGTGAA	54	NM_001007479.1 (Liu et al., 2020)
GAPDH	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	73	NM_204305.1 (Liu et al., 2020)
TAF2	GCCATGGCTCTTCTGAGAGAT	AGAGTTGGCTAGGGCATCAA	150	NM_001305162.1 (Mazanko et al., 2019)
TAF4	CCAACTTGACTGCATTAGCTGC	TCGCGTAAACTGTCTGGTTGT	146	XM_417400.6 (Mazanko et al., 2019)

The Olfm4 and Ki67 primers were designed by Primer Express 3.0 (Thermo Fisher Scientific).

Table 3-2. Effect of probiotics on BW and BWG in broiler chickens from doh to d8.

	BW (g)					BWG (g)			
Treatment	doh	d2	d4	d6	d8	doh - d2	d2 - d4	d4 - d6	d6 - d8
Control	45.6	56.5	86.0	125.4	171.6	11.0	29.5[a]	39.8	46.5
Probiotic 1	45.1	57.4	83.3	121.3	169.3	12.3	26.1[b]	38.0	47.7
Probiotic 2	45.3	56.1	82.5	121.4	167.4	10.8	26.8[b]	38.9	46.0
SEM	0.50	0.73	0.95	1.37	2.08	0.49	0.43	0.88	1.11
P value	0.83	0.47	0.052	0.09	0.39	0.13	0.0003[*]	0.41	0.58

All individual chickens were weighed at doh, d2, d4, d6, and d8. The experimental unit is cage (n = 6).

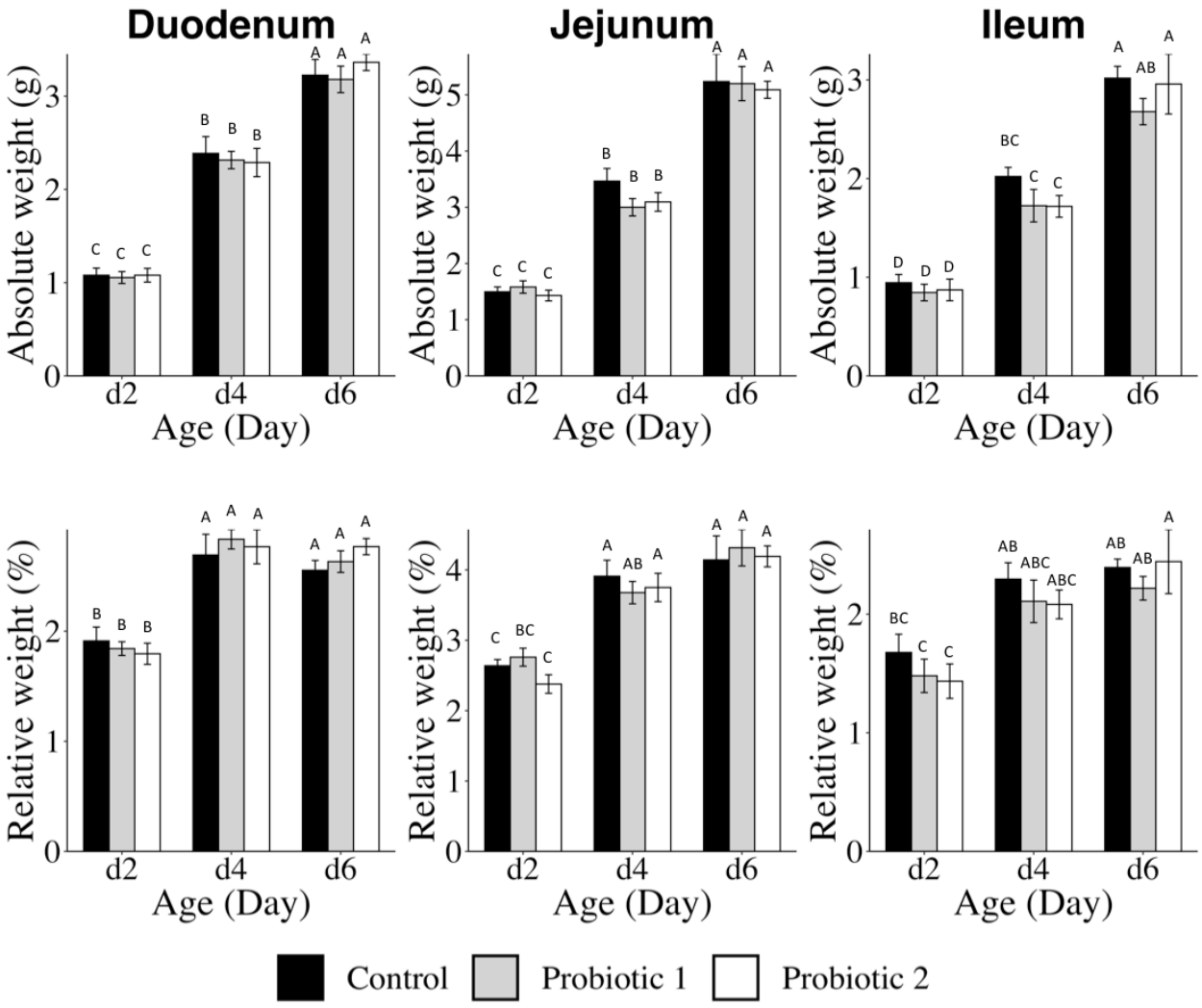


Figure 3-1. Effect of probiotics on intestinal absolute weight (upper panel) and intestinal relative weight (absolute weight/body weight, lower panel) in broiler chickens (n = 6). Bars show mean ± individual SE. 2-way ANOVA and Tukey's test were used for data analysis from d2 to d6. Significant differences are indicated with different letters ($P < 0.05$).

Table 3-3. Effect of probiotics on intestinal morphology of duodenum, jejunum, and ileum in broiler chickens.

Segment[1]	Duodenum			Jejunum			Ileum		
Gene	VH	CD	VH/CD	VH	CD	VH/CD	VH	CD	VH/CD
Treatment									
Control	1109.6	108.3	11.1	631.2	112.5	5.8	474.8	90.5^{a}	5.5
Probiotic 1	1177.8	111.2	11.4	725.1	110.8	6.7	488.4	89.9^{a}	5.6
Probiotic 2	1110.5	100.2	11.5	688.8	114.3	6.2	458.9	78.9^{b}	6.1
SEM	24.51	5.37	0.67	44.26	4.5	0.37	13.64	2.86	0.22
P-value	0.09	0.33	0.9	0.33	0.86	0.27	0.32	0.01*	0.17
Age									
d2	901.1^{c}	88^{b}	11^{ab}	511.7^{b}	93.8^{b}	5.6^{b}	361^{c}	80^{b}	4.7^{b}
d4	1162.3^{b}	121.5^{a}	10.1^{b}	710.9^{a}	126.3^{a}	5.8^{b}	502^{b}	96.6^{a}	5.4^{b}
d6	1334.5^{a}	110.2^{a}	12.8^{a}	822.6^{a}	117.5^{a}	7.3^{a}	559^{a}	82.7^{b}	7.1^{a}
SEM	24.51	5.37	0.67	44.26	4.5	0.37	13.64	2.86	0.22
P-value	<0.0001*	0.0003*	0.02*	<0.0001*	<0.0001*	0.005*	<0.0001*	0.0004*	<0.0001*
Interaction									
T×A	0.27	0.8	1	0.41	0.78	0.53	0.48	0.07	0.04*

[1] Two-way ANOVA and Tukey's test were used for analysis of significant differences (n = 5). Significant differences were indicated as different letters ($P < 0.05$).

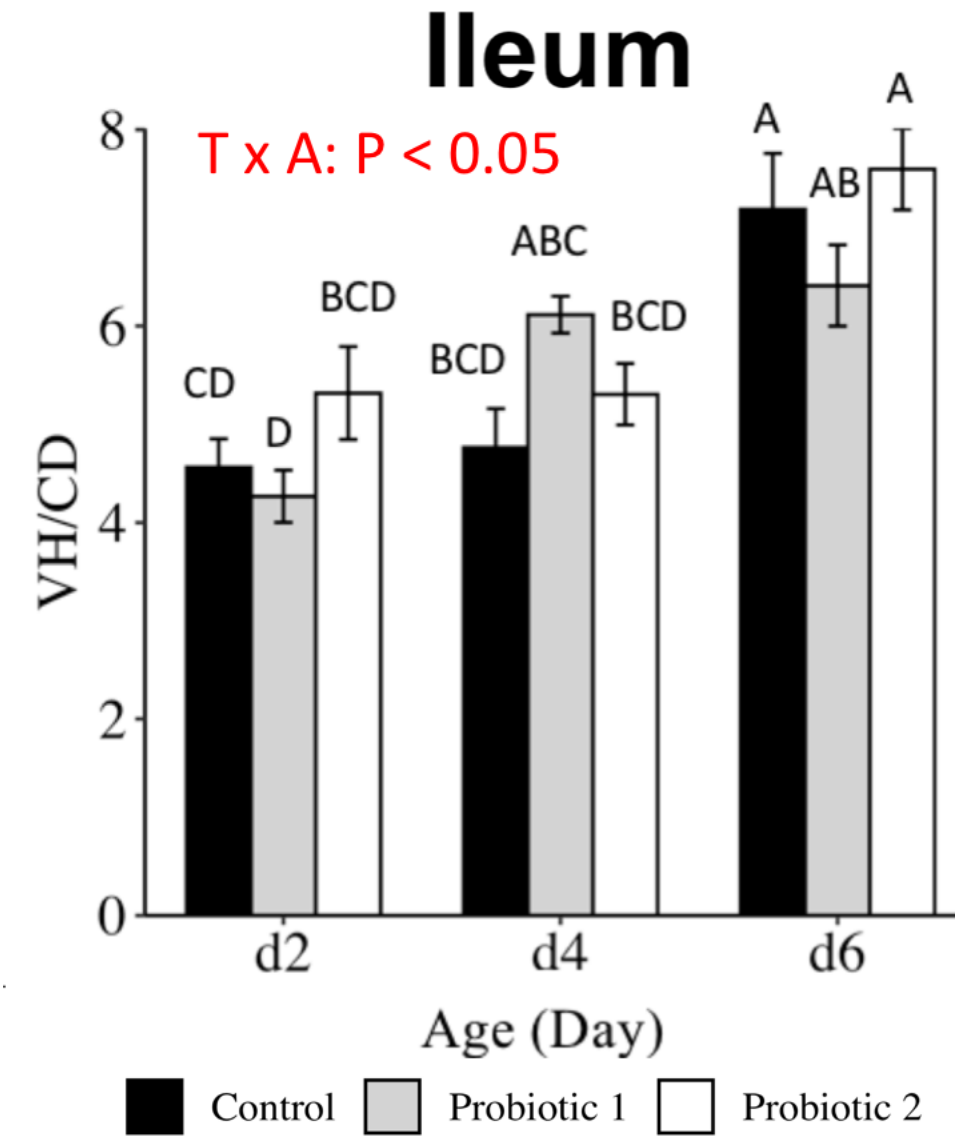


Figure 3-2. Interaction between treatment and age for VH/CD in the ileum of broiler chickens (n = 5). 2-way ANOVA and Tukey's test were used for analysis of data. Bars show mean ± individual SE. Significant differences were indicated as different letters ($P < 0.05$).

Table 3-4. Effect of probiotics on the mRNA expression of marker genes for intestinal epithelial cells in duodenum (n = 6).

Segment[1]	Duodenum				
Gene	Olfm4	Ki67	PepT1	Muc2	LEAP2
Treatment					
Control	1.8	0.9	0.9^{a}	1.6	1.5
Probiotic 1	1.2	0.8	0.5^{b}	1.6	1.8
Probiotic 2	1.4	0.8	0.8^{ab}	1.6	1.9
SEM	0.27	0.08	0.08	0.13	0.2
P-value	0.23	0.26	0.003*	0.94	0.67
Age					
d2	0.9^{b}	0.9^{a}	0.9	1.1^{b}	1.1^{b}
d4	1.7^{ab}	0.6^{b}	0.6	1.8^{a}	1.2^{b}
d6	1.9^{a}	0.9^{a}	0.7	2^{a}	2.9^{a}
SEM	0.27	0.08	0.08	0.13	0.2
P-value	0.001*	0.0006*	0.47	<0.0001*	<0.0001*
Sex					
Male	1.4	0.8	0.8^{a}	1.5	1.7
Female	1.7	0.8	0.5^{b}	1.8	1.7
SEM	0.22	0.06	0.07	0.11	0.16
P-value	0.2	0.99	0.003*	0.27	1
Interaction					
T×A	0.74	0.047*	0.1	0.69	0.0006*
T×S	0.61	0.58	0.36	0.37	0.73
A×S	0.2	0.22	0.55	0.68	0.04*
T×A×S	0.41	0.98	0.21	0.46	0.35

[1]Three factorial analysis and Tukey's test were used for analysis. Significance test was performed after log10 transformation. Significant differences were indicated as difference letters and asterisk.

Table 3-5. Effect of probiotics on the mRNA expression of marker genes for intestinal epithelial cells in jejunum (n = 6).

Segment[1]	Jejunum				
Gene	Olfm4	Ki67	PepT1	Muc2	LEAP2
Treatment					
Control	1.3	1.3^{b}	0.8	1.5	0.7^{a}
Probiotic 1	1	1.3^{ab}	0.6	1.7	0.4^{b}
Probiotic 2	0.8	1.6^{a}	0.8	1.5	0.3^{b}
SEM	0.26	0.09	0.14	0.12	0.07
P-value	0.65	0.007*	0.38	0.45	0.0002*
Age					
d2	1.4	1.1^{b}	1.1	1.2^{b}	0.8^{a}
d4	0.9	1.4^{ab}	0.6	1.7^{a}	0.3^{c}
d6	0.9	1.7^{a}	0.6	1.9^{a}	0.3^{b}
SEM	0.26	0.09	0.14	0.12	0.07
P-value	0.78	0.002*	0.18	0.0003*	<0.0001*
Sex					
Male	1	1.4	0.9	1.5	0.5
Female	1.1	1.3	0.6	1.7	0.4
SEM	0.21	0.08	0.11	0.1	0.05
P-value	0.84	0.3	0.09	0.4	0.51
Interaction					
T×A	0.34	<0.0001*	0.36	0.98	<0.0001*
T×S	0.97	0.37	0.73	0.66	0.12
A×S	0.59	0.28	0.55	0.96	0.17
T×A×S	0.69	0.35	0.1	0.59	0.42

[1] Three factorial analysis and Tukey's test were used for analysis. Significance test was performed after log10 transformation. Significant differences were indicated as difference letters and asterisk.

Table 3-6. Effect of probiotics on the mRNA expression of marker genes for intestinal epithelial cells in ileum (n = 6).

Segment[1]	Ileum				
Gene	Olfm4	Ki67	PepT1	Muc2	LEAP2
Treatment					
Control	1.8	0.9[b]	1.3	2.4	2
Probiotic 1	1.9	1.1[a]	1.1	2.6	1.9
Probiotic 2	1.8	1[ab]	1.4	3.1	2.6
SEM	0.26	0.04	0.15	0.2	0.62
P-value	0.76	0.018[*]	0.42	0.49	0.61
Age					
d2	1.5[b]	1.1[a]	1.2[a]	1.6[c]	1.5
d4	2.4[a]	0.8[b]	1.5[a]	2.5[b]	2.4
d6	1.6[ab]	1.1[a]	1.1[a]	4.1[a]	2.7
SEM	0.26	0.04	0.15	0.2	0.62
P-value	0.04[*]	0.0002[*]	0.04[*]	<0.0001[*]	0.79
Sex					
Male	1.7	1	1.5	2.5	1.8
Female	2	1	1	3	2.7
SEM	0.21	0.03	0.12	0.16	0.5
P-value	0.52	0.31	0.06	0.43	0.31
Interaction					
T×A	0.48	0.14	0.15	0.83	0.97
T×S	0.73	0.41	0.5	0.8	0.78
A×S	0.77	0.32	0.65	0.84	0.56
T×A×S	0.22	0.06	0.15	0.59	0.73

[1] Three factorial analysis and Tukey's test were used for analysis. Significance test was performed after log10 transformation. Significant differences were indicated as difference letters and asterisk.

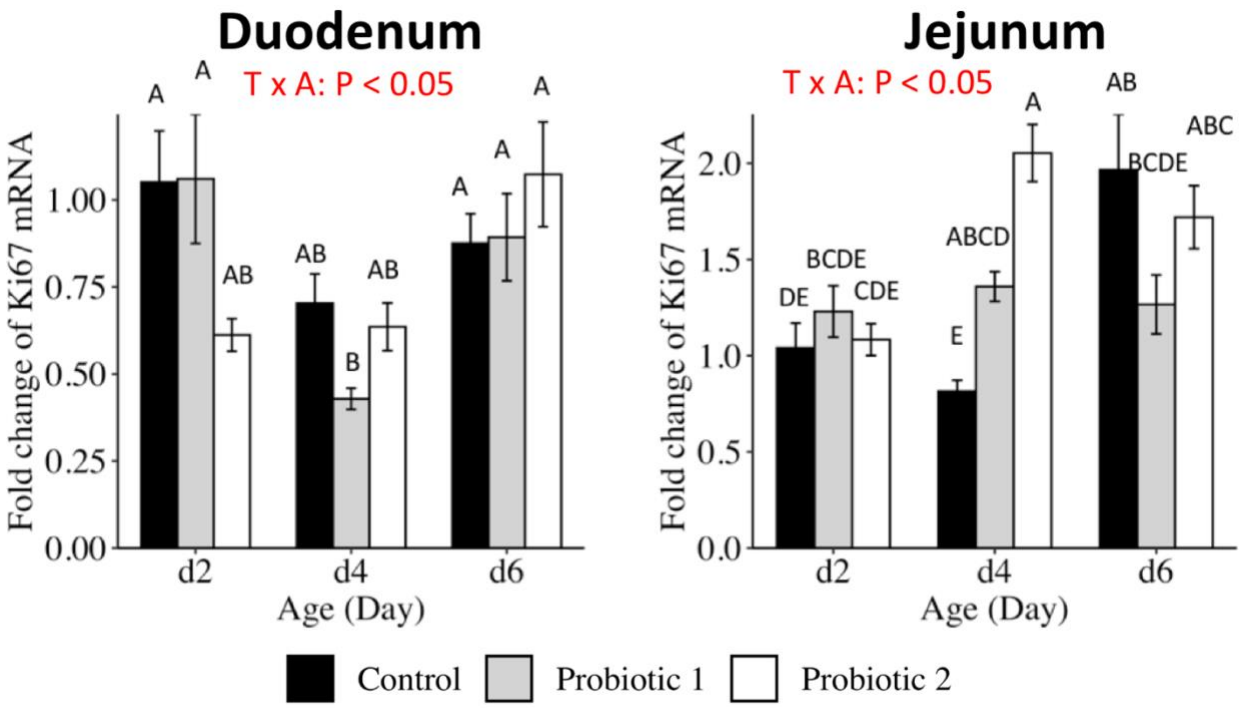


Figure 3-3. Interaction between treatment and age for Ki67 mRNA abundance in the duodenum and jejunum of broiler chickens (n = 6). The fold change of marker genes at d2, d4, and d6 was calculated using the $2^{-\Delta\Delta Ct}$ method. The Control at d2 was used as the calibrator. Bars show mean ± individual SE. Significance test was performed after log10 transformation. Tukey's test was used for statistical analysis. Significant differences are indicated with different letters ($P < 0.05$).

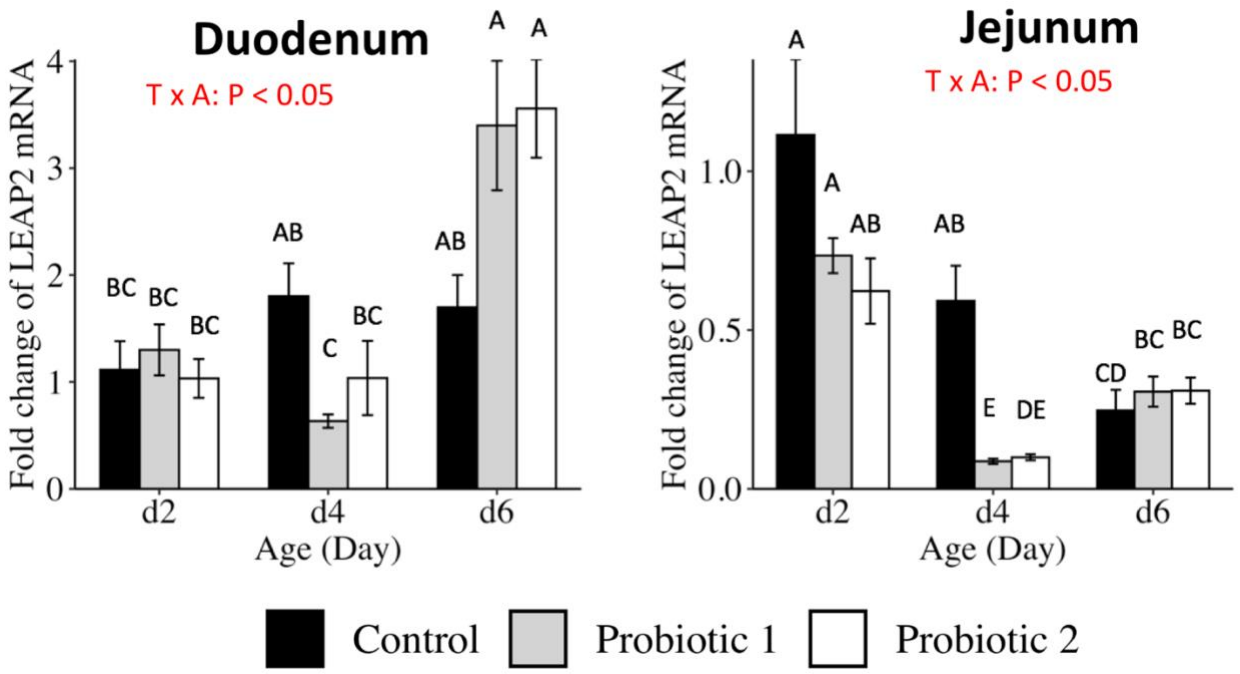


Figure 3-4. Interaction between treatment and age for LEAP2 mRNA abundance in the duodenum and jejunum of broiler chickens (n = 6). The fold change of marker genes at d2, d4, and d6 was calculated using the $2^{-\Delta\Delta Ct}$ method. The Control at d2 was used as the calibrator. Bars show mean ± individual SE. Significance test was performed after log10 transformation. Tukey's test was used for statistical analysis. Significant differences are indicated with different letters ($P < 0.05$).

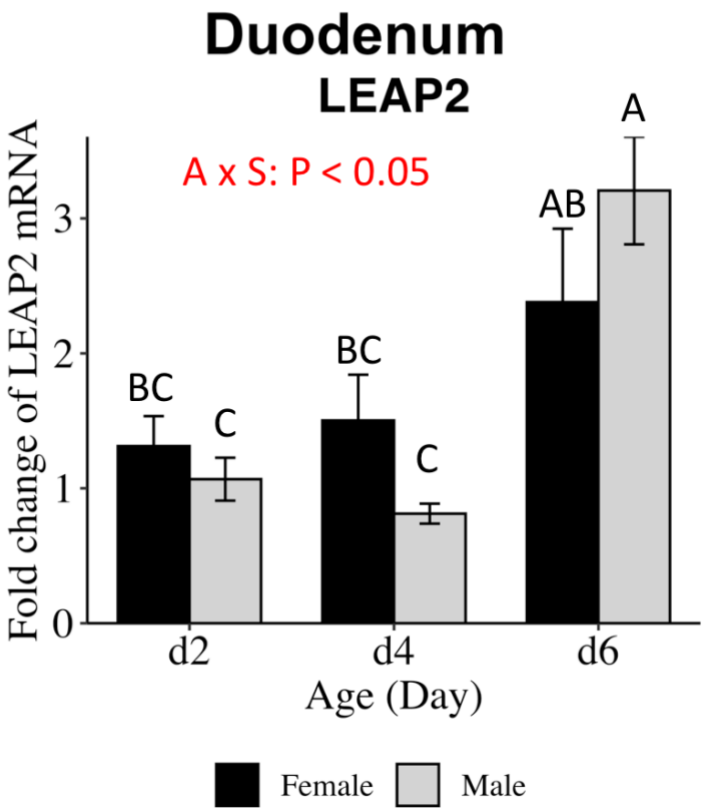


Figure 3-5. Interaction between age and sex for LEAP2 mRNA abundance in the duodenum of broiler chickens (n = 6). The fold change of marker genes at d2, d4, and d6 was calculated using the $2^{-\Delta\Delta Ct}$ method. The Control at d2 was used as the calibrator. Bars show mean ± individual SE. Significance test was performed after log10 transformation. Tukey's test was used for statistical analysis. Significant differences are indicated with different letters ($P < 0.05$).

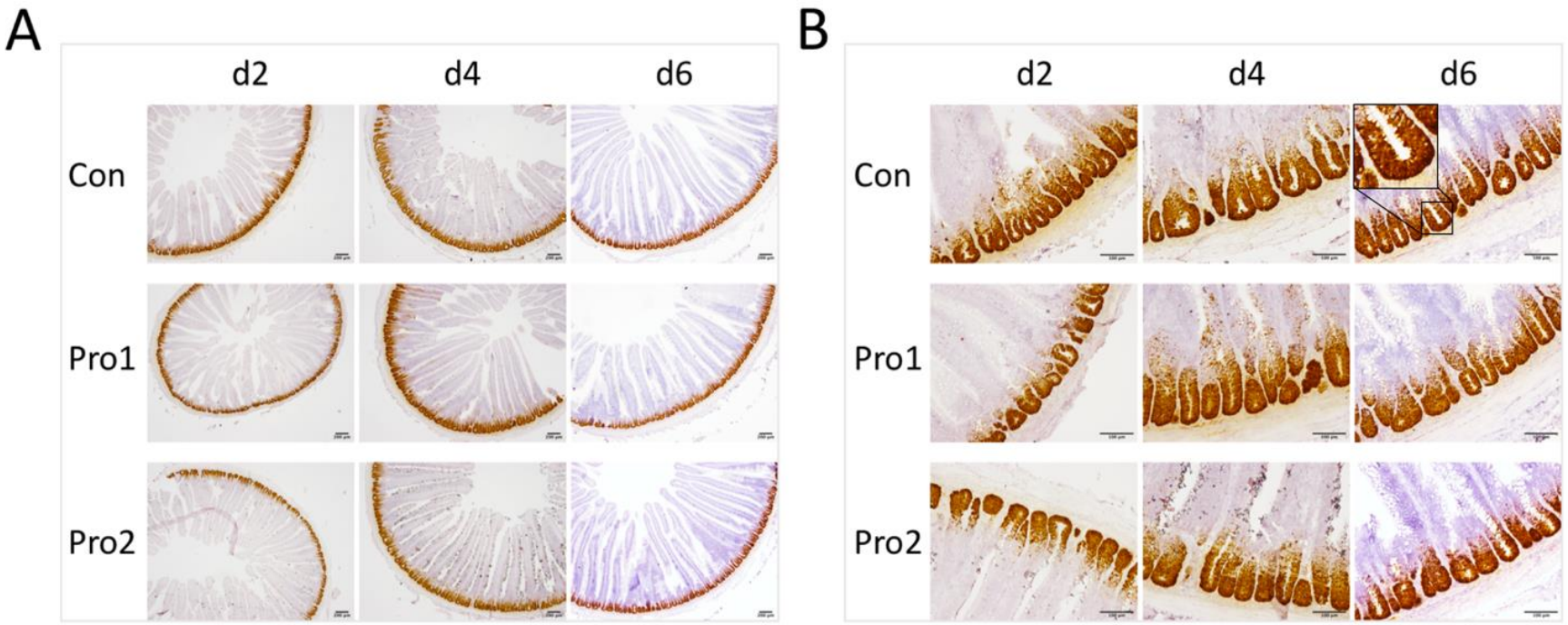


Figure 3-6. Effect of probiotics on expression of Olfm4 mRNA in the duodenum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Duodenal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n=5) were analyzed by in situ hybridization using a probe for olfactomedin 4 (brown stain). Slides were counterstained with hematoxylin. Images of Olfm4 mRNA in the duodenum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Inset in the Control at d6 shows a magnified image of a crypt.

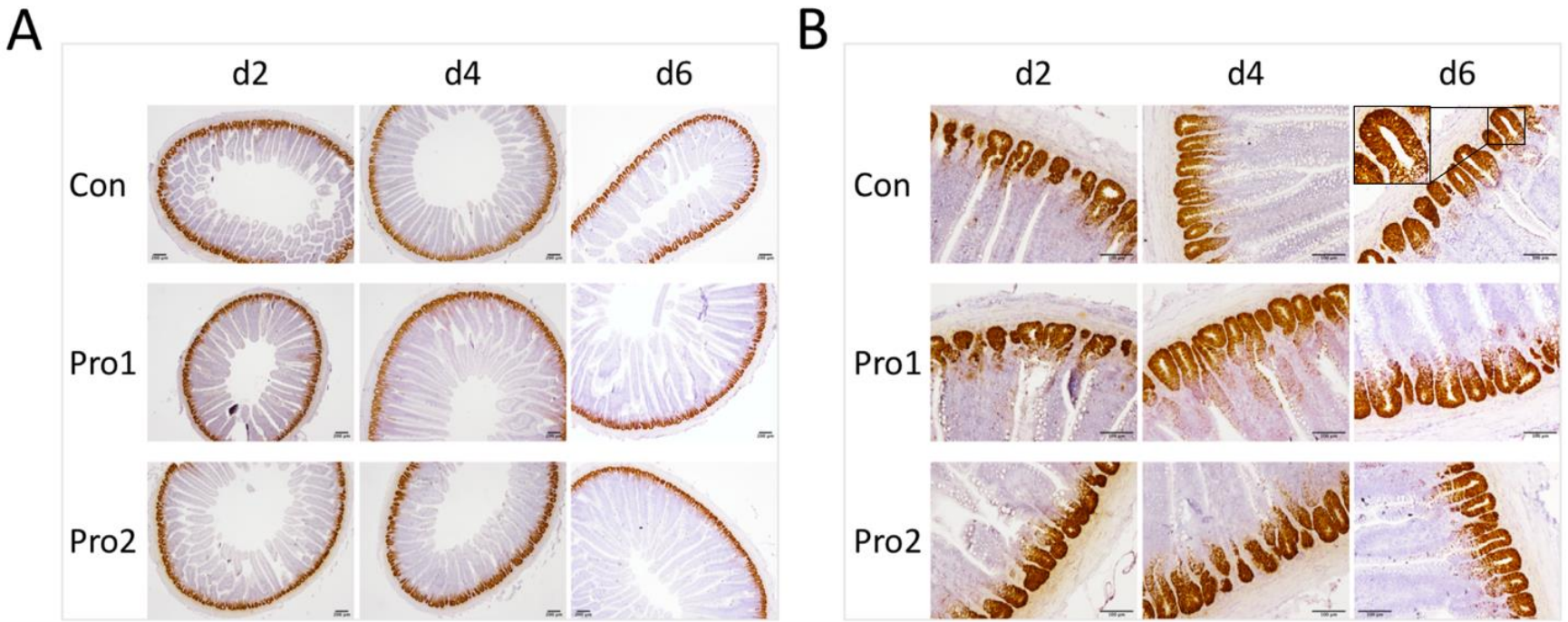


Figure 3-7. Effect of probiotics on expression of Olfm4 mRNA in the jejunum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Jejunal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n=5) were analyzed by in situ hybridization using a probe for olfactomedin 4 (brown stain). Slides were counterstained with hematoxylin. Images of Olfm4 mRNA in the jejunum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Inset in the Control at d6 shows a magnified image of a crypt.

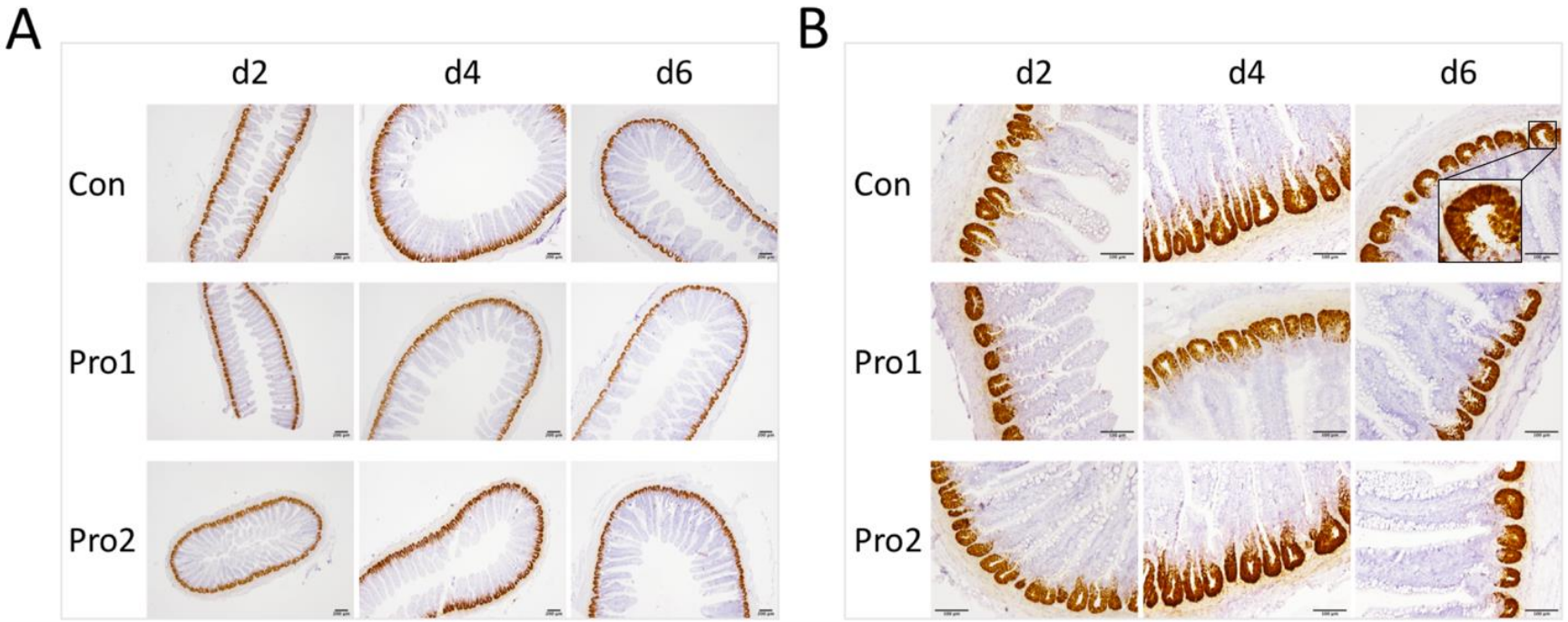


Figure 3-8. Effect of probiotics on expression of Olfm4 mRNA in the ileum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Ileal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n=5) were analyzed by in situ hybridization using a probe for olfactomedin 4 (brown stain). Slides were counterstained with hematoxylin. Images of Olfm4 mRNA in the ileum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Inset in the Control at d6 shows a magnified image of a crypt.

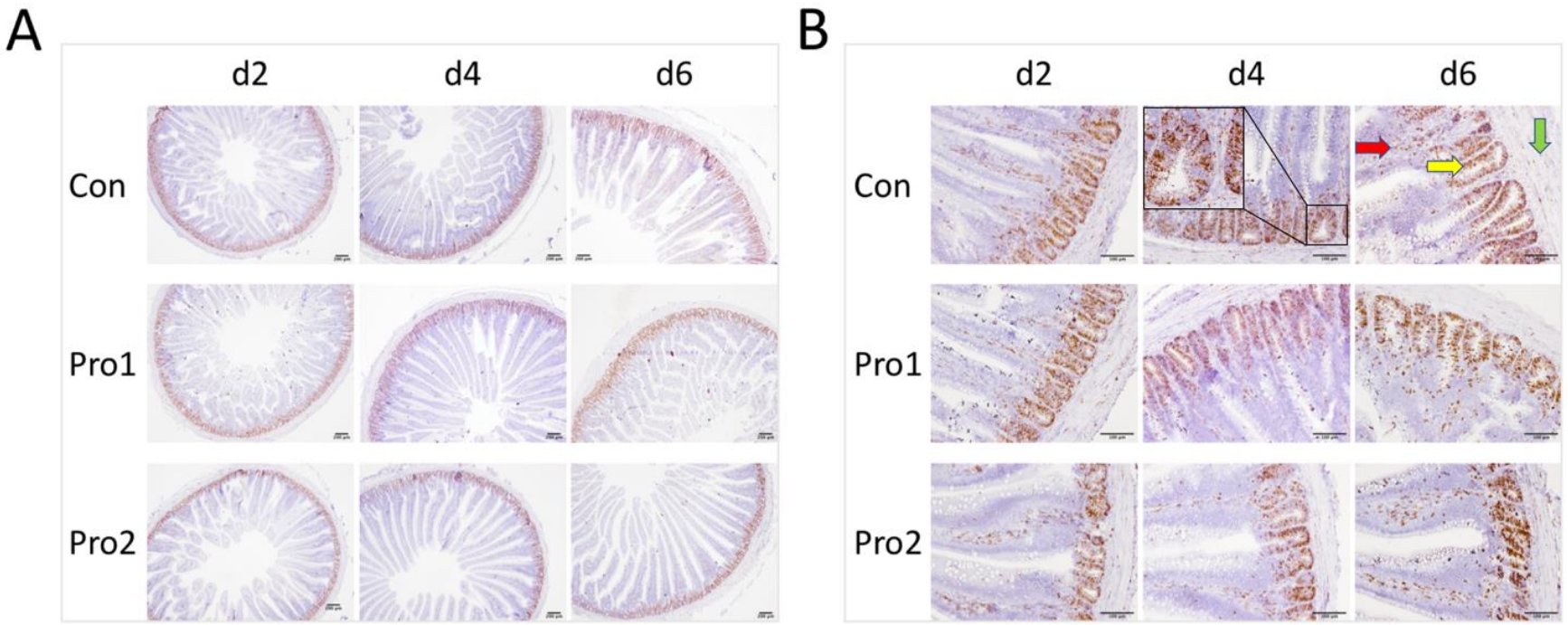


Figure 3-9. Effect of probiotics on expression of Ki67 mRNA in the duodenum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Duodenal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n=5) were analyzed by in situ hybridization using a probe for Ki67 (brown stain). Slides were counterstained with hematoxylin. Images of Ki67 mRNA in the duodenum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Yellow arrow refers to the crypt; red arrow refers to the middle of the villi; green arrow refers to the muscularis. Inset in the Control at d4 shows a magnified image of a crypt.

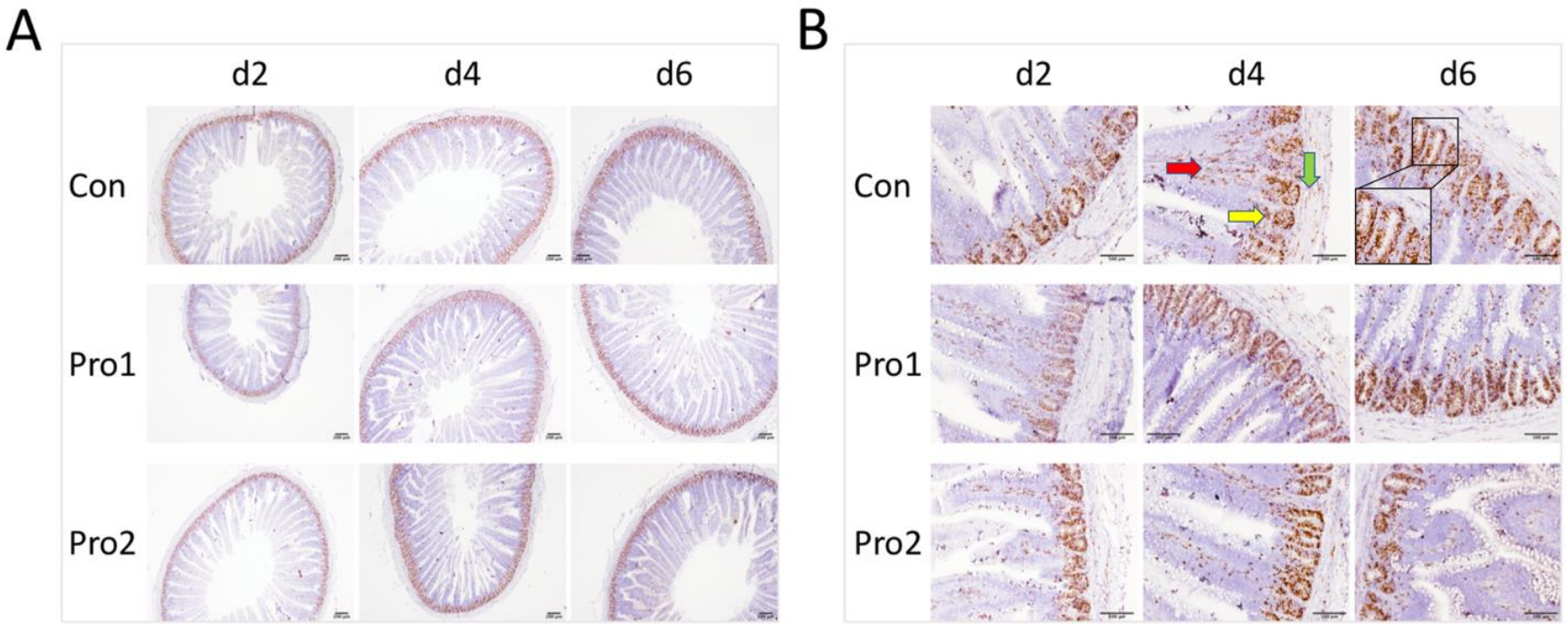


Figure 3-10. Effect of probiotics on expression of Ki67 mRNA in the jejunum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Jejunal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n=5) were analyzed by in situ hybridization using a probe for Ki67 (brown stain). Slides were counterstained with hematoxylin. Images of Ki67 mRNA in the jejunum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Yellow arrow refers to the crypt; red arrow refers to the middle of the villi; green arrow refers to the muscularis. Inset in the Control at d6 shows a magnified image of a crypt.

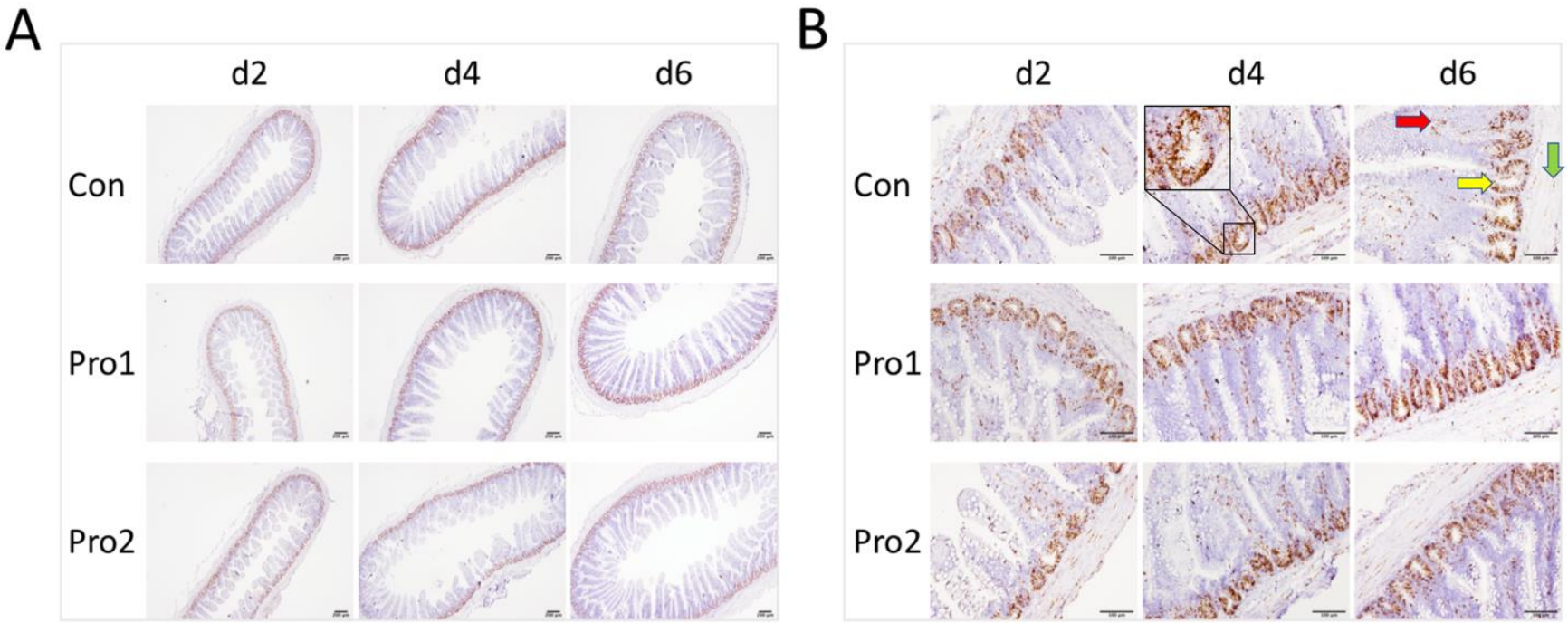


Figure 3-11. Effect of probiotics on expression of Ki67 mRNA in the ileum. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Ileal samples were collected at day 2 (d2), day 4 (d4) and day 6 (d6). Formalin fixed paraffin embedded sections (n = 5) were analyzed by in situ hybridization using a probe for Ki67 (brown stain). Slides were counterstained with hematoxylin. Images of Ki67 mRNA in the ileum for the Control (Con), Probiotic 1 (Pro1), and Probiotic 2 (Pro2) at d2, d4, and d6 are shown at (A) 40 × magnification (scale bar represents 200 μm) and (B) at 200 × magnification (scale bar represents 100 μm). Yellow arrow refers to the crypt; red arrow refers to the middle of the villi; green arrow refers to the muscularis. Inset in the Control at d4 shows a magnified image of a crypt.

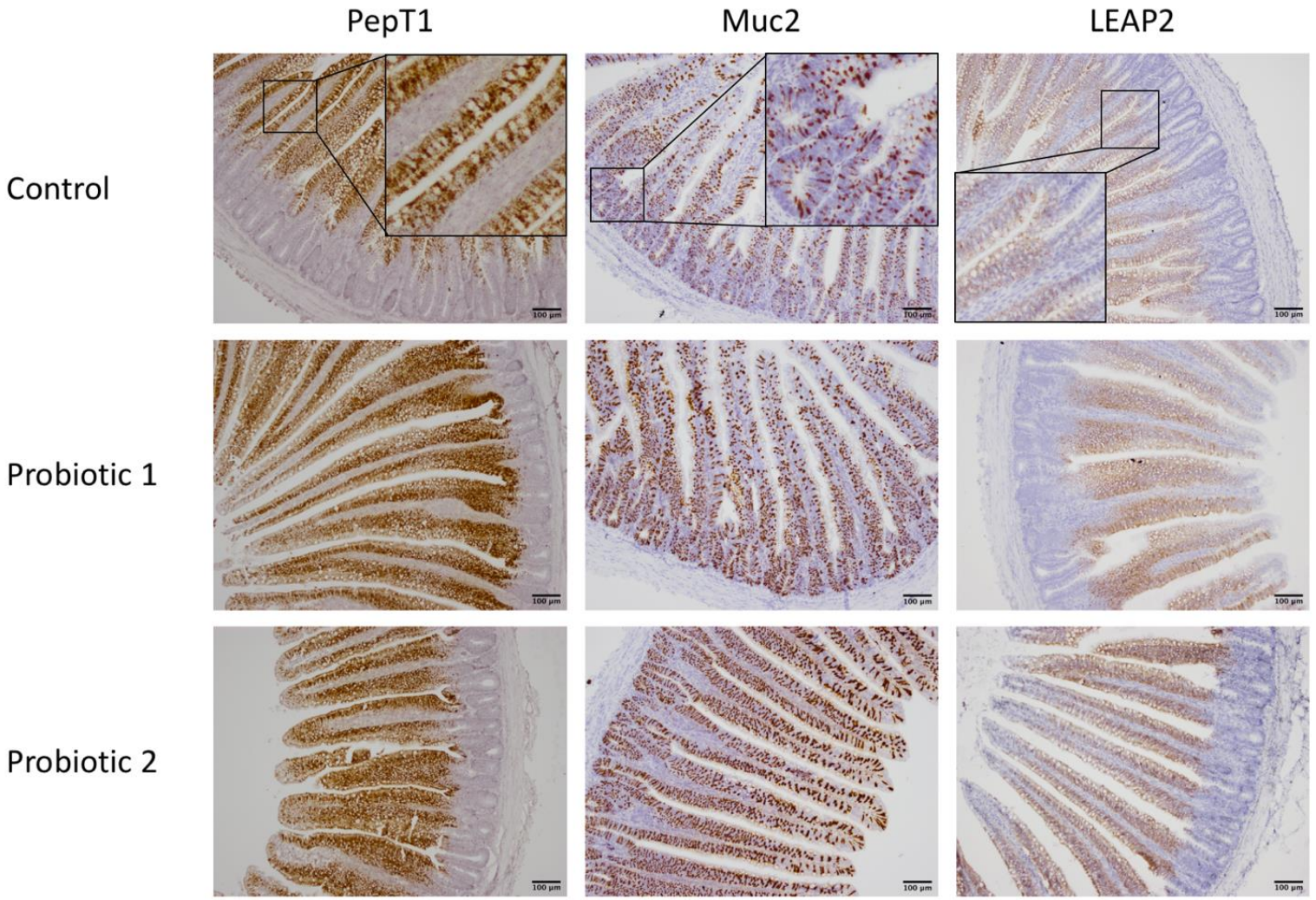
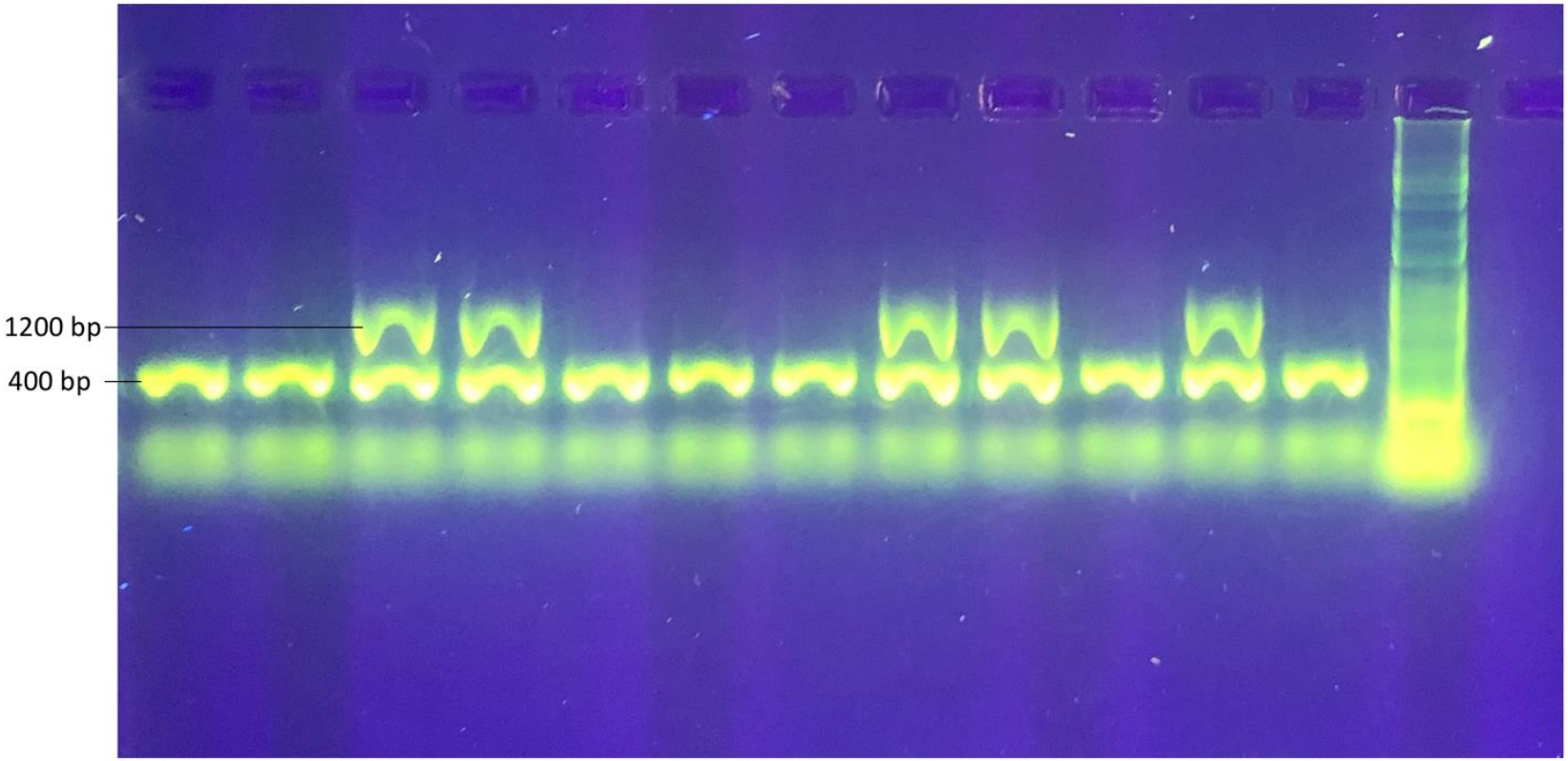


Figure 3-12. Effect of probiotics on expression of PepT1, Muc2, and LEAP2 mRNA in the jejunum at d4. Chicks were fed a control diet or diets supplemented with two different probiotics from day of hatch until day 6. Formalin fixed paraffin embedded sections (n=5) at day 4 were analyzed by in situ hybridization using a probe for PepT1, Muc2, and LEAP2 (brown stain). Slides were counterstained with hematoxylin. Images were captured at 100 × magnification. Scale bar represents 100 μm. Insets in the Control show a magnified image of a villus.



Supplement 1. Sex determination by PCR for collected chicks in this study. Male chicks (ZZ) exhibit only 1 band at 400 bp (tyrosinase). Female chicks (ZW) exhibit 2 bands at 400 bp (tyrosinase) and 1200bp (W chromosome).

Chapter IV Effect of probiotics on tight junction proteins in the small intestine of young chicks

Abstract

Tight junction **(TJ)** proteins form a paracellular barrier to prevent intestinal pathogens from entering the blood circulation and to regulate the permeability of ions, water and solutes in the small intestine. In this study, the effect of probiotics on the mRNA abundance for the TJ proteins claudin 1 (CLDN1), occludin (OCLN), zonula occludens 1 (ZO1) and junctional adhesion molecule 2 (JAM2) were investigated in the small intestine of young broiler chickens. The chicks were randomly placed into 3 treatments: basal diet without probiotics (Control) and two basal diets supplemented with different probiotics (Probiotic 1 and Probiotic 2). At day (d) 2, d4, and d6, one chick from each replicate cage was randomly selected for collection of the duodenum, jejunum, and ileum. A piece from the middle of each segment was fixed for paraffin embedding and in situ hybridization analysis. An additional piece was minced and stored at -80 ℃ for gene expression analysis. Three factorial analysis (treatment × age × sex) and Tukey's test were used for statistical analysis. Significance was set at $P < 0.05$. There were no significant 3-way (T × A × S) or 2-way (T × A, T × S, and A × S) interactions for mRNA expression of all the selected TJ proteins. Probiotic treatment did not affect mRNA abundance of tight junction proteins in all three intestinal segments. However, mRNA abundance of tight junctions in the small intestine were affected by age except ZO1 in the jejunum. CLDN1 and JAM2 were decreased by age in all three segments, while OCLN mRNA was increased by age. Altering the expression of tight junction proteins with age may change the composition or structure of the tight junctions. OCLN mRNA in the duodenum of female chicks had a greater mRNA abundance than males, which might result in tighter tight

junctions. In situ hybridization revealed that OCLN mRNA was expressed in the epithelial cells of both the villus and crypt in all three small intestinal segments.

Keywords: Tight junction proteins, probiotics, small intestine, in situ hybridization, chicken.

Introduction

The tight junction (TJ) is an essential structure between two adjacent endodermal, epithelial, or myelinated cells (González-Mariscal et al., 2003) and is located from the apical to lateral membranes of cells (Farquhar and Palade, 1963). The TJ forms a physical barrier to prevent the passage of pathogens and limit paracellular transportation of ions (Ballard et al., 1995; Awad et al., 2017). Besides its role as a permeability barrier, the TJ is also involved in cell polarity, epithelial cell growth and differentiation (Schneeberger and Lynch, 2004). The TJ of endodermal cells and epithelial cells consists of three major transmembrane protein complexes, such as occludin (OCLN), claudins (CLDNs), junction adhesion molecules (JAMs), and zonula occludens (ZOs). The former two are tetra-span membrane proteins that form the backbone of the TJ and JAMs are single span transmembrane proteins that regulate TJ protein formation (Awad et al., 2017). ZO1 links the transmembrane TJ proteins to the actin cytoskeleton and recruits cytosolic molecules involved in cell signaling (Schneeberger and Lynch, 2004).

Tight junction proteins are important in the small intestine as a physical barrier to prevent luminal pathogens from entering the circulation (Slifer and Blikslager, 2020). Expression of ZO1, OCLN, CLDNs and JAMs was reported in the small intestinal epithelium of chickens (Haworth et al., 2005; Ozden et al., 2010; Tabler et al., 2020). In pathological conditions, enteric pathogens and their toxins can disrupt the TJ directly by proteolytic degradation of specific TJ proteins or change the distribution of specific TJ proteins, or indirectly by altering cytoskeleton rearrangement (Berkes et al., 2003). Heat stress also affected TJ protein expression and subsequently increased

intestinal paracellular permeability, which was reversed by probiotic treatment in chickens (Song et al., 2013; Tabler et al., 2020).

Probiotics are a type of beneficial bacteria that consist of various strains to promote growth performance. In recent studies, probiotics alleviated the damage to the lumen and fixed the integrity of the gut caused by pathogens by increasing TJ protein expression (Emami et al., 2019; Memon et al., 2020). The probiotic containing *Bacillus subtilis* increased body weight gain and enhanced intestinal integrity in the jejunum of necrotic enteritis challenged chickens by increasing CLDN1 and JAM2 expression (Keerqin et al., 2021). *Salmonella enterica* infection decreased OCLN mRNA in the jejunum and ileum; however, a multi-strain probiotic supplement including *Lactobacillus acidophilus*, *L. fermentum*, *Pediococcus acidilactici,* and *L. casei*, significantly increased OCLN and ZO1 in the ileum (Chang et al., 2020). Broiler chickens heat-stressed from d22 to d42 reduced OCLN and ZO1 mRNA expression with a decrease of transepithelial electrical resistance compared to the thermoneutral group. A probiotic mixture (*Bacillus subtilis*, *Bacillus licheniformis*, and *Lactobacillus plantarum*) increased OCLN mRNA expression in the jejunum (Song et al., 2014).

There are few studies that looked at the effect of probiotics on TJ proteins within the first week post-hatch. The objective of this study was to investigate the effect of probiotics on the mRNA abundance of TJ proteins (OCLN, ZO1, CLDN1, JAM2) in the small intestine of broiler chicks. Intestinal cells expressing OCLN mRNA were also identified using in situ hybridization.

Materials and Methods

Animals and tissue collection

Cobb 500 broiler chicks (n = 204) were hatched and housed at the poultry farm at Virginia Tech. At day of hatch (doh), 6 chicks were euthanized by cervical dislocation for collection of

duodenum, jejunum, and ileum. The remaining 198 chicks were randomly separated into 3 dietary groups: Control group (basal diet without probiotic), Probiotic 1 (basal diet with 0.5 g/kg PoultryStar® synbiotic product), Probiotic 2 (basal diet supplemented with 1.5 g/kg PrimaLac). Each group had 6 replicate cages with 11 chicks per cage. Chicks had ad libitum access to water and feed. At day (d) 2, d4, and d6, one chick from each replicate cage was randomly selected for collection of 3 segments of the small intestine. An approximately 3 cm piece from the middle of each segment was fixed in 10% buffered formalin for 24 h, dehydrated in 70% ethanol for 24 h, and then transferred into fresh 70% ethanol for shipment to StageBio (Mount Jackson, VA) for paraffin embedding. Additional pieces of each segment were minced, frozen on dry ice, and stored at -80 ℃ for gene expression analysis.

RNAscope in situ hybridization

Paraffin embedded blocks (n = 3) were cut into 5 µm sections. In situ hybridization (ISH) was performed with a custom chicken occludin (OCLN) probe, using the RNAscope™ 2.5 HD Assay - RED kit (Advanced Cell Diagnostics, Newark, CA) following the manual from the company. The sections were counterstained with 50% Gill I hematoxylin solution. Images were captured using a Nikon DS-Ri1 camera on a Nikon Eclipse 80i microscope.

RNA extraction, cDNA synthesis, and real time qPCR

Total RNA was extracted from frozen small intestinal segments using TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) and the Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA). One µg of total mRNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Real time quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-time PCR system (Thermo Fisher Scientific) and the default cycling conditions: 95 °C for 20 s, 40

cycles of 90 °C for 3 s followed by 60 °C for 30 s. Each well contained 1.5 µl cDNA diluted 1:20, 5 µl Fast SYBR Green Master Mix (Thermo Fisher Scientific), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), and 1.5 µl DEPC water. Each sample was duplicated on the same qPCR plate. The primers for the TJ proteins are listed in Table 4-1. Ribosomal protein L4 (RPL4), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA-box-binding protein-associated factor 4 (TAF4) were selected as the three most stable reference genes for this study using the GeNorm program in qbase+ (Hellemans et al., 2007).

Statistical analysis

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The geometric mean of three reference genes was used to calculate ΔCt. The calibrator for each gene was the average ΔCt in the Control group at d2. Because of the non-normal distribution of the data, log10 transformation, which was the single transformation method that best fit all of the tested genes, was used before statistical analysis. Three factorial analysis (treatment × age × sex) and Tukey's test were used for statistical analysis, with significance set as $P < 0.05$.

Results

There was no significant interaction among three factors (T × A × S) or between two factors (T × A, T × S, and A × S); however, the mRNA abundance of all TJ proteins was affected by age with the exception of ZO1 in the jejunum (Table 4-2). ZO1 was decreased from d4 to d6 in the duodenum, while it was increased from d2 to d4 in the ileum. OCLN mRNA abundance increased from d2 to d4 and to d6 in the duodenum, from d4 to d6 in the jejunum, and from d2 to d6 in the ileum. In contrast to the pattern with OCLN, CLDN1 and JAM2 decreased with age in the duodenum, jejunum, and ileum. CLDN1 mRNA abundance decreased from d2 to d4 and to d6 in

the duodenum and ileum, and decreased from d2 to d4 or to d6 in the jejunum. JAM2 mRNA decreased from d4 to d6 in the duodenum, and from d2 to d6 in the jejunum and ileum.

Tight junction mRNA was not affected by treatments in all the three segments. However, there was a main effect of sex for OCLN mRNA in the duodenum. OCLN mRNA abundance was greater in the duodenum of female chickens compared with males.

The distribution of OCLN mRNA at doh, d2, d4, and d6 was visualized by RNAscope in situ hybridization (Figures 4-1, 4-2 and 4-3). OCLN mRNA was localized to the epithelial cells in both the crypt and villi of the duodenum, jejunum, and ileum. There was no OCLN mRNA signal in the submucosa and muscularis layer. At doh, the OCLN mRNA signal had greater intensity in the bottom region than the tip of the villi in all three segments of the small intestine. However, from d2 to d6, the intensity of OCLN in the bottom of the villi had not clear difference from the tip of villi.

Discussion

The TJ is an important structure between adjacent cells. It forms a physical barrier to prevent pathogens from passing between the intestinal epithelial cells (Zeisel et al., 2019). The expression level of tight junction proteins in the small intestine was altered in pathogenic states like coccidiosis or necrotic enteritis. A higher dose of a mixed *Eimeria* challenge including *E. maxima*, *E. acervulina*, and *E. tenella* increased CLDN1 and JAM2 mRNA, but not OCLN and ZO2 mRNA in the jejunum of broilers at day 6 post infection (Teng et al., 2020). *Salmonella* Typhimurium infection decreased the ZO1 mRNA in the ceca of broilers, while OCLN and CLDN1 mRNA did not change. However, the decrease of ZO1 mRNA was reversed by the supplementation of *Lactobacillus plantarum* (Wang et al., 2018). Supplementation of the commercial probiotic Primalac to necrotic enteritis-challenged chicks improved the jejunal

epithelial barrier by increasing OCLN3 mRNA compared with the control group (Emami et al., 2019). These studies suggested that mRNA expression of TJ proteins in the small intestine was readily affected by pathogens, while probiotics prevented the decrease of TJ protein mRNA, and subsequently prevented the loss of intestinal integrity.

Probiotics supplementation can also increase tight junction protein expression in healthy chickens. The mRNA expression of OCLN was upregulated in the ileum of broilers supplemented with *Bacillus subtilis* strain 1781 for 14 d. However, a probiotic mixture containing *B. subtilis* strain 1104 and strain 747 increased ZO1, OCLN, and JAM2 mRNA, and a combination of *B. subtilis* strain 1781 and strain 747 increased ZO1 and JAM2 mRNA in the ileum (Gadde et al., 2017). After supplementation with *Paenibacillus polymyxa 10* for 21 d, OCLN and CLDN1 mRNA were increased in the ileum of broiler chicks, while *Lactobacillus plantarum 16* supplementation did not significantly increase OCLN and CLDN1 mRNA in the ileum (Wu et al., 2019a). These results indicated that probiotics were able to increase the mRNA expression of tight junction proteins, and this effect was dependent upon the probiotic strains. Wu et al. (2019b) showed that pretreatment with *Enterococcus faecium* upregulated CLDN1 protein expression compared with the non-supplemented control in the jejunum of broilers. In this study, probiotic treatment did not upregulate the mRNA abundance of TJ proteins in the small intestine. This is not surprising because this was only a short-term study of chicks fed probiotics from doh to d6.

The mRNA abundance of tight junction proteins was affected with age. In this study, OCLN mRNA was upregulated with age, while OCLN1 and JAM2 mRNA were decreased with age. Proszkowiec-Weglarz et al. (2020) reported that CLDN1 was decreased from d2 to d4 in the jejunum and from d4 to d6 post hatch in the ileum for the group that received water and feed immediately. However, OCLN mRNA in the ileum was increased from d2 to d6 post hatch for the

immediately fed group. In a recent study, CLDN1 mRNA was downregulated in the jejunum and cecum of broiler chickens at d7 and d14 compared with d1 (Von Buchholz et al., 2021). This shows that mRNA expression of different TJ proteins can increase or decrease with age, which may change the composition or structure of the TJ.

OCLN mRNA abundance in the duodenum can be affected by sex. OCLN is a tetra-span tight junction protein, which plays an important role to maintain intestinal integrity. Exposure to stimuli such as inflammation shifted the location of OCLN protein to the cytoplasm, which subsequently cause an increase in intestinal epithelial permeability (Von Buchholz et al., 2021). The greater OCLN mRNA abundance in duodenum of female chickens in this study may suggest that the duodenal integrity in the female chickens was greater than males. This result is consistence to the finding of Goo et al. (2019), who reported that increasing stocking density increased the serum LPS concentration in male chicken, but did not affect the serum LPS concentration in female chickens. The increase of serum LPS concentration indicates an increasing intestinal permeability. Their results might suggest that female chickens had greater intestinal integrity than males, even though there was no effect of sex for tight junction mRNA and TER (trans-epithelial electrical resistance) value in the jejunum of broilers.

In this study, OCLN mRNA was located in both the crypt and villus of the small intestine in broilers. This result is consistent with a previous study in mice that showed that OCLN was located in endodermal cells and epithelial cells. Mouse OCLN mRNA was expressed in intestinal organoids enriched in stem cells, goblet cells, Paneth cells, and enterocytes (Pearce et al., 2018). The OCLN mRNA expression in the stem and Paneth cell organoids was around twice that of the enterocyte and goblet cell organoids. In mammals, intestinal stem cells and Paneth cells are located in the crypt, while enterocytes and goblet cells are located along the villus. Thus, higher expression

level in the stem cell organoid may explain our results on doh, during which OCLN mRNA signal intensity in the bottom of the villi was greater than in the top of the villi. This may suggest that the small intestinal integrity in the crypt and bottom of the villi is greater than the tip of the villi.

Summary and Conclusion

In this study, probiotics did not affect mRNA abundance of the tight junction proteins in all three intestinal segments. However, tight junction mRNA was affected by age, which might change the composition or structure of the tight junctions in the small intestine within 1-wk-post-hatch. OCLN mRNA was greater in the duodenum of female chicken than males, which might cause tighter tight junctions. OCLN mRNA was distributed in the small intestinal crypt and villi of the duodenum, jejunum, and ileum.

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Table 4-1: Primers for qPCR.

Genes	Forward Primer sequence (5′-3′)	Reverse Primer Sequence (5′-3′)	Product (bp)	Accession number
ZO1[1]	TGTAGCCACAGCAAGAGGTG	CTGGAATGGCTCCTTGTGGT	98	XM_015278975.2
OCLN[2]	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCGTCTTCTGG	240	NM 205128.1
CLDN1[2]	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA	115	NM 001013611.2
JAM2[3]	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA	59	NM_001006257.1
RPLP1[4]	TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG	62	NM_205322.1
RPL4[4]	TCAAGGCGCCCATTCG	TGCGCAGGTTGGTGTGAA	54	NM_001007479.1
GAPDH[4]	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	73	NM_204305.1
TAF2[5]	GCCATGGCTCTTCTGAGAGAT	AGAGTTGGCTAGGGCATCAA	150	NM_001305162.1
TAF4[5]	CCAACTTGACTGCATTAGCTGC	TCGCGTAAACTGTCTGGTTGT	146	XM_417400.6

Primers are from: [1]Li et al., 2015; [2]Shao et al., 2013; [3]Chen et al., 2015; [4]Liu et al., 2020; [5]Mazanko et al., 2019.

Table 4-2: Effect of probiotics on the mRNA abundance of tight junction proteins in the small intestine of broiler chicks.

Segment	Duodenum				Jejunum				Ileum			
Gene	ZO1	OCLN	CLDN1	JAM2	ZO1	OCLN	CLDN1	JAM2	ZO1	OCLN	CLDN1	JAM2
Treatment												
Control	1.2	2.8	0.6	0.8	1.3	1.9	0.8	0.9	1.2	3.6	0.6	0.7[a]
Probiotic 1	1.1	3.1	0.6	0.8	1.5	2.5	0.7	1	1.2	4.5	0.7	0.7[a]
Probiotic 2	1.2	3.3	0.8	0.7	1.3	2.4	1	1	1.2	4.4	0.8	0.5[a]
SEM	0.1	0.77	0.13	0.07	0.11	0.53	0.26	0.1	0.08	1.02	0.09	0.06
P-value	0.91	0.49	0.22	0.59	0.2	0.8	0.36	0.52	0.22	0.62	0.18	0.04*
Age												
d2	1.1	1.3[b]	1.3[a]	1[a]	1.2	1.5[b]	1.6[a]	1.2[a]	1.1[b]	2.5[b]	1.2[a]	0.8[a]
d4	1.4	2.7[b]	0.5[b]	0.8[a]	1.5	1.9[b]	0.5[b]	0.9[ab]	1.4[a]	3.2[ab]	0.6[b]	0.6[ab]
d6	1.1	5.1[a]	0.2[c]	0.5[b]	1.3	3.4[a]	0.5[b]	0.8[b]	1.2[ab]	6.8[a]	0.2[c]	0.5[b]
SEM	0.1	0.77	0.13	0.07	0.11	0.53	0.26	0.1	0.08	1.02	0.09	0.06
P-value	0.08	<0.0001*	<0.0001*	0.0006*	0.39	0.012*	0.0001*	0.005*	0.03*	0.02*	<0.0001*	0.007*
Sex												
Male	1.1	2.5[b]	0.7	0.7	1.3	2	0.8	1	1.2	5.2	0.7	0.6
Female	1.3	3.9[a]	0.7	0.8	1.3	2.7	0.9	0.9	1.2	3.5	0.6	0.7
SEM	0.08	0.63	0.1	0.06	0.09	0.43	0.21	0.08	0.07	0.83	0.07	0.05
P-value	0.11	0.04*	0.96	0.12	0.86	0.16	0.66	0.72	0.84	0.15	0.43	0.86
Interaction												
T×A	0.69	0.52	0.44	0.98	0.89	0.92	0.61	0.8	0.19	0.98	0.29	0.33
T×S	0.26	0.49	0.93	0.93	0.19	0.97	0.57	0.14	0.11	0.87	0.51	0.15
A×S	0.24	0.43	0.93	0.08	0.41	0.86	0.54	0.33	0.08	0.56	0.86	0.38
T×A×S	0.85	1	0.17	0.35	0.45	0.62	0.84	0.2	0.33	0.96	0.89	0.49

[1] Three factorial analysis and Tukey's test were used to analyze significant differences (n = 6). Different letters indicate significant differences within a gene ($P < 0.05$).

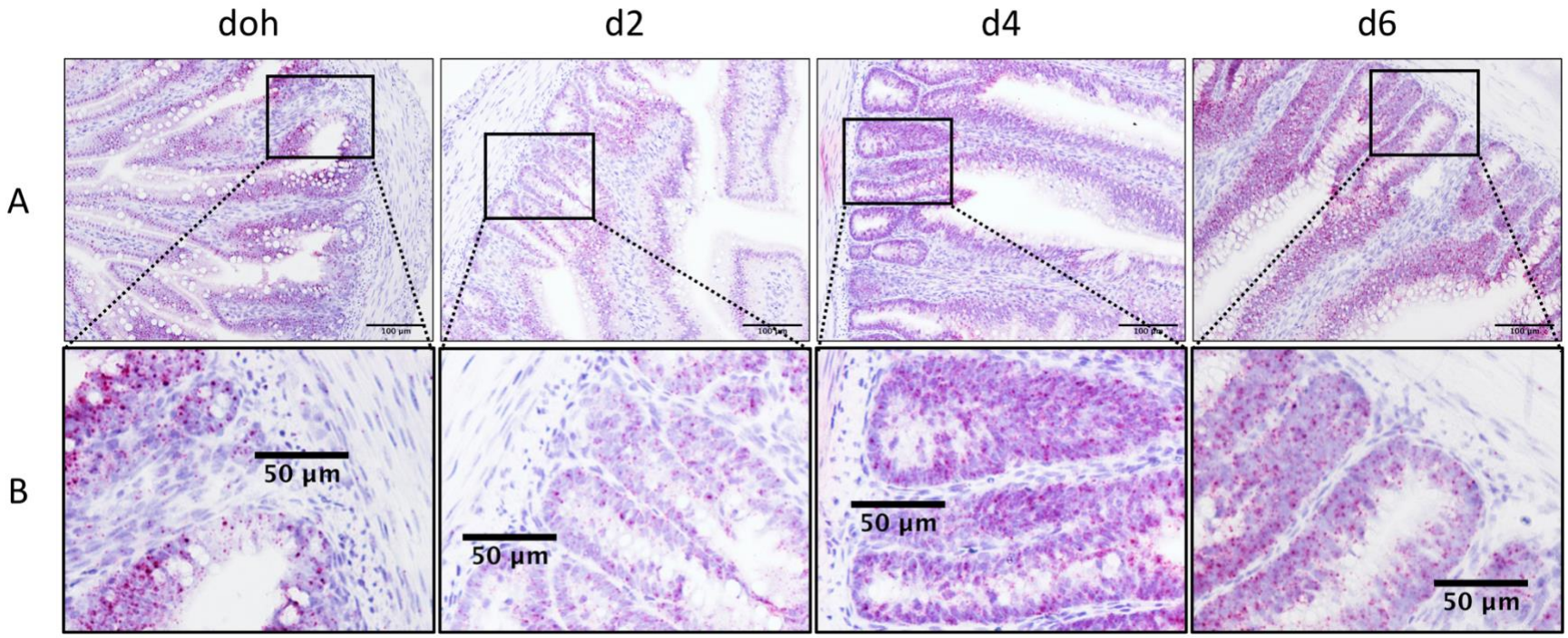

Figure 4-1. Distribution of Occludin (OCLN) mRNA in the duodenum of broiler chicks fed without probiotics. Duodenal samples were collected at day of hatch (doh), day 2 (d2), day 4 (d4), and day 6 (d6), and fixed in 10% buffered formalin. In situ hybridization was performed with a chicken OCLN probe (red signal) on paraffin embedded sections (5 μm, n = 3). Hematoxylin was used for counterstaining. A. OCLN mRNA distribution in the duodenum at 200 × magnification. The scale bar represents 100 μm. B. Enlarged images showing OCLN mRNA distribution in the crypt.

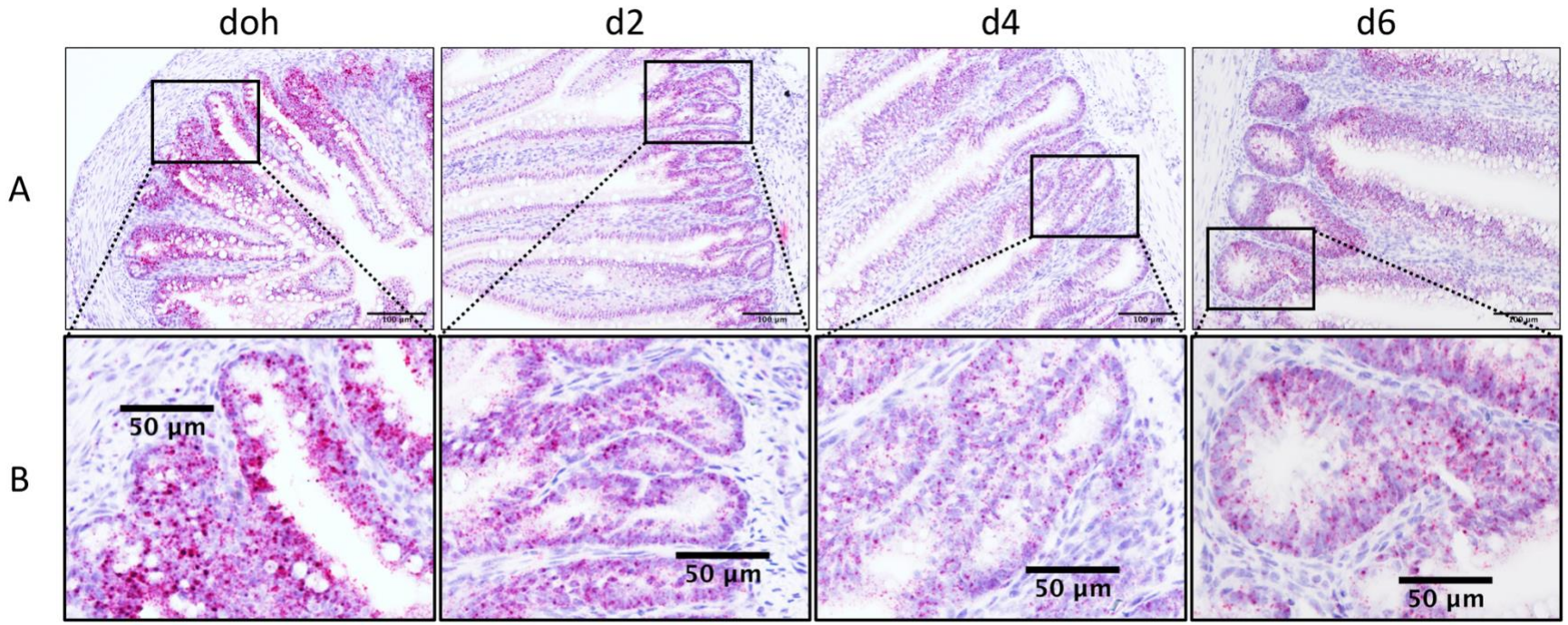

Figure 4-2. Distribution of Occludin (OCLN) mRNA in the jejunum of broiler chicks fed without probiotics. Jejunal samples were collected at day of hatch (doh), day 2 (d2), day 4 (d4), and day 6 (d6), and fixed in 10% buffered formalin. In situ hybridization was performed with a chicken OCLN probe (red signal) on paraffin embedded sections (5 μm, n = 3). Hematoxylin was used for counterstaining. A. OCLN mRNA distribution in the jejunum at 200 × magnification. The scale bar represents 100 μm. B. Enlarged images showing OCLN mRNA distribution in the crypt.

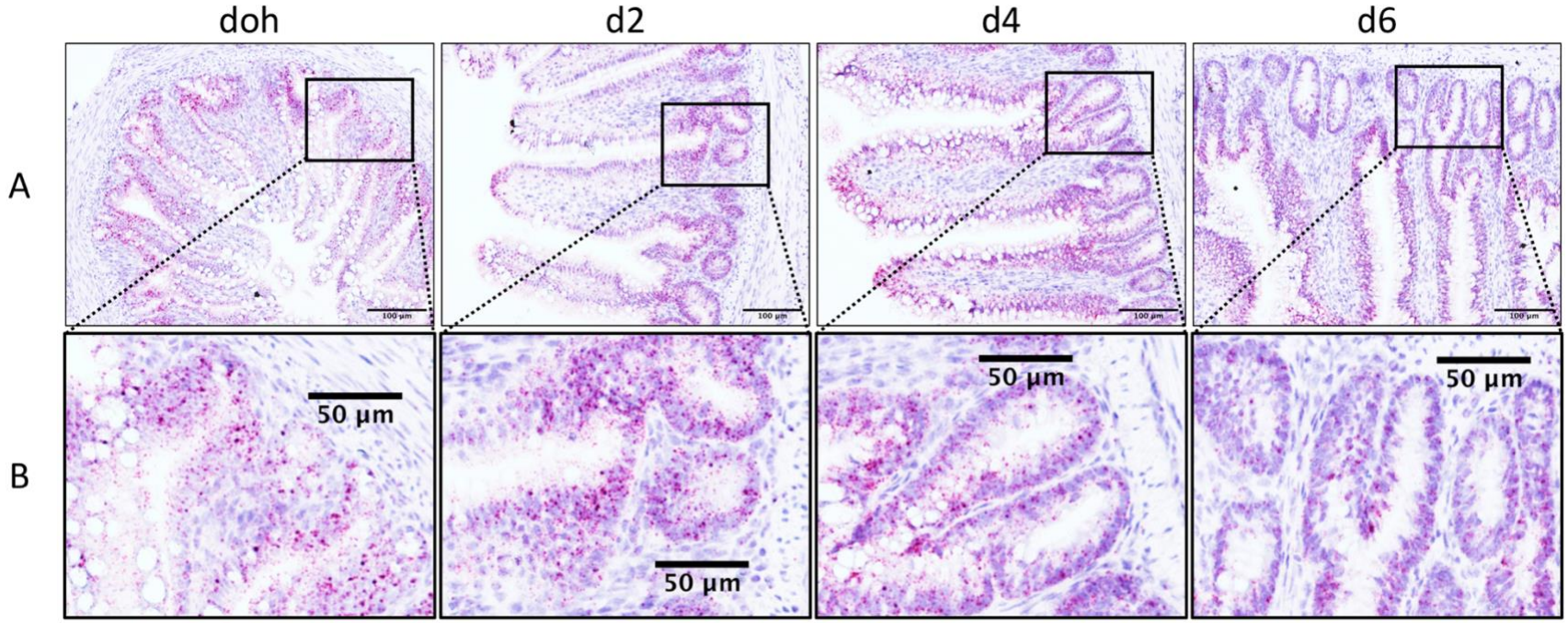

Figure 4-3. Distribution of Occludin (OCLN) mRNA in the ileum of broiler chicks fed without probiotics. Ileal samples were collected at day of hatch (doh), day 2 (d2), day 4 (d4), and day 6 (d6), and fixed in 10% buffered formalin. In situ hybridization was performed with a chicken OCLN probe (red signal) on paraffin embedded sections (5 μm, n = 3). Hematoxylin was used for counterstaining. A. OCLN mRNA distribution in the ileum at 200 × magnification. The scale bar represents 100 μm. B. Enlarged images showing OCLN mRNA distribution in the crypt.

Chapter V Effects of high incubation temperature on tight junction proteins in the yolk sac of embryonic broilers

Abstract

The yolk sac (YS) is a multifunctional organ that is involved in nutrient uptake and serves as a biological barrier between the embryo and yolk contents. Tight junction proteins form tight junction complexes between adjacent epithelial or endothelial cells. The objective of this study was to investigate the effect of heat stress on gene expression of tight junction proteins in the YS of broiler chickens. For experiment 1, on embryonic day 12 (E12), eggs were randomly assigned into 2 incubators with normal temperature (37.5 ℃) or a hyperthermal environment (39.5 ℃). The YS samples were collected every other day from E7 to DOH. The effect of high incubation temperature on mRNA abundance of tight junction proteins was determined within day from E13 to DOH by t-test. In order to obtain fresh samples for in situ hybridization analysis and to replicate the qPCR results at the normal temperature, a second experiment was conducted to collect YS samples from E7 to DOH at only 37.5 ℃. Expression of tight junction mRNA was analyzed by one-way ANOVA and Tukey's test. Significance was set at $P < 0.05$ for both experiments. High incubation temperature upregulated mRNA abundance of ZO1 on E13, and JAMA and HSP90 mRNA abundance on E17, but decreased JAMA mRNA abundance on E19 and OCLN mRNA abundance on DOH. All tested tight junction protein mRNA were expressed greater during the early (E7) than the middle (E13 to E15) stages. These results indicate that high incubation temperature initially upregulates the mRNA abundance of tight junction proteins in the YS of embryonic broilers during short term exposure, but reduces tight junction protein mRNA abundance after long-term heat exposure.

Keywords: high incubation temperature, tight junction protein, heat shock protein, yolk sac, chicken.

Introduction

The YS is an extra-embryonic membrane that extends from the embryonic intestine. It is a multifunctional organ involved in nutrient uptake, immunity, and hematopoiesis (Yadgary et al., 2011; Guedes et al., 2014; Zhang and Wong, 2017, 2019; Wong and Uni, 2021). The YS plays an important role for the developing chicken embryo, for not only nutrient absorption from the yolk but also as a physiological barrier to protect the embryo from pathogens (Zhang and Wong, 2019). However, experimental infections with *Escherichia coli* or *Salmonella* Enteritidis at embryonic day 12 caused hyperemia, inflammation, and abnormal endodermal epithelial cells of the YS, which were highly associated with embryonic mortality (Rezaee et al., 2020). This showed that the integrity of the YS is very important for chicks.

The tight junction is one of the elements of the epithelial junction complex (tight junction, adherens junction, gap junction, and desmosomes), which is located in the apical lateral membrane (Farquhar and Palade, 1963; Zihni et al., 2016). The tight junction functionally seals the extraembryonic compartment to maintain the integrity of the YS and prevents the intracellular passage of yolk components (Mobbs and McMillan, 1979). In other tissues, the tight junction contains a series of transmembrane proteins, including tetra-span proteins (such as occludin and claudins) and single-span transmembrane proteins (mostly junction adhesion molecules). Zonula occludens protein 1 (ZO1) was the first identified tight junction protein (Stevenson et al., 1986; Ulluwishewa et al., 2011). ZO-1 interacts with different transmembrane proteins via different PDZ (PSD95-DlgA-ZO1 homology) domains and regulates cytoskeleton reorganization (Fanning et al., 1998; Itoh et al., 1999, 2001; Utepbergenov et al., 2006; Ulluwishewa et al., 2011). Tight junction

proteins form a physiological barrier and play an essential role in the regulation of paracellular transport of ions and water (Van Itallie and Anderson, 2004). The presence of tight junction proteins in the YS of poultry has not been reported.

Heat stress generates behavioral, physiological, and immunological responses for animals, which subsequently decrease their productivity (Lara and Rostagno, 2013). A recent study showed that the gastrointestinal tract responds to heat stress in broiler chickens (Rostagno, 2020). Exposure to high temperature increased blood flow to the body surface and reduced blood flow to internal organs, including the intestine (Van Wijck et al., 2012; Rostagno, 2020). This reduced oxygen and nutrient supply to the intestinal epithelium subsequently decreased the turnover of enterocytes (Hall et al., 1999; Lambert, 2009; Rostagno, 2020). Furthermore, intestinal ischemia causes a "leaky" intestinal epithelium and endotoxemia. Evidence from a rat study showed that heat stress increased intestinal permeability to FITC-dextran 4 and induced significant intestinal epithelial damage with loss of microvilli (Lambert et al., 2002; Lambert, 2009). Varasteh et al. (2015) demonstrated that heat stress altered the expression of tight junction proteins in the gastrointestinal tract and impaired the intestinal barrier integrity, which facilitated the entry of pathogens or luminal antigens through the intestinal epithelium.

Heat stress during incubation dramatically decreased hatchability and performance in the post-hatch stages of broiler chickens (Narinç et al., 2016). However, there is no available information regarding the response of tight junction proteins to high incubation temperature in the YS of embryonic broiler chickens. Thus, the objective of this study was to examine the effect of high temperature incubation on mRNA abundance of zonula occludens-1 (ZO1), occludin (OCLN), claudin-1 (CLDN1), junctional adhesion molecule A (JAMA), and junctional adhesion molecule

2 (JAM2) as well as heat shock protein 70 (HSP70) and HSP90 in the YS of embryonic broiler chickens.

Materials and methods

Experiment 1: Effect of high incubation temperature on tight junction protein expression in the yolk sac

Egg incubation and tissue collection

The YS samples for Experiment 1 were from a previous study in our lab that investigated the effect of high incubation temperature on endodermal nutrient transporters in the YS of broiler embryos (Reynolds, 2019). In that study, Reynolds (2019) reported that high incubation temperature (39.5 °C,) dramatically decreased hatchability (42%) of broiler eggs compared to hatchability (93%) at optimal thermoneutral temperature (37.5 °C). A description of the methods from that study are as follows. 468 Cobb 500 broiler eggs were obtained from a local commercial hatchery and incubated at 37.5 °C at the Virginia Tech poultry center. At embryonic (E) day 7, E9, and E11, 6 eggs were randomly selected for YS sampling after euthanasia of the embryo. At embryonic day 12 (E12), all eggs were candled, and infertile eggs were culled and 110 fertile eggs were transferred to an incubator set at 39.5 °C until day of hatch (DOH). The remaining 282 fertile eggs were continually incubated at 37.5 °C. At E13, E15, E17, E19, and DOH, 6 eggs from each treatment were randomly selected and the embryo was euthanized by cervical dislocation. The YS samples were rinsed in cold 1X phosphate-buffered saline (1 × PBS) to remove the residual yolk. Two pieces of the YS (2 - 3 cm^2 each) from each bird were fixed in 10% neutral-buffered formalin for 24 h at room temperature (RT), transferred into vials filled with 70% ethanol for 24 h at RT, then stored in fresh 70% ethanol at 4 ℃. These samples were shipped to StageBio (Mount Jackson, VA) for paraffin embedding, which were then used for RNAscope in-situ hybridization. One or

two pieces of the remaining YS were minced, rapidly frozen on dry ice and stored at -80 °C for subsequent gene expression analysis. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RNA extraction and gene expression analysis

Frozen YS samples (n = 6 for each treatment, around 25 mg) from E7 to DOH were homogenized in 450 ul TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) with a Tissue Lyser II (Qiagen, Hilden, Germany). Total RNA was extracted following the instructions of the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). The concentration and purity of RNA were assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). cDNA samples were reverse transcribed starting with 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and then diluted 1:20 with DEPC water for real time quantitative PCR (qPCR). A 10 μl qPCR reaction in each well consisted of 5 μl of Fast SYBR Green Master mix (Thermo Fisher Scientific), 1.5 μl of diluted cDNA sample, 1 μl of forward primer (5 μM) and 1 μl of reverse primer (5 μM), and 1.5 μl of DEPC water. qPCR was performed using an Applied Biosystems 7500 Fast Real-time PCR system (Thermo Fisher Scientific) with the default fast program: 95 °C for 20 s, 40 cycles of 90 °C for 3 s and 60 °C for 30 s. Each sample was run in duplicate. The mRNA abundance of selected tight junction (TJ) proteins including ZO1, OCLN, CLDN1, JAMA, JAM2, and heat shock protein 70 (HSP70) and HSP90 were determined using the primers shown in Table 5-1. The stability of reference genes such as ribosomal RNA, ribosomal protein lateral stalk subunit P0 and P1 (RPLP0 and RPLP1) using all samples was analyzed using RefFinder (Xie et al., 2012). Only RPLP0 and RPLP1 were used as reference genes in this study due to their higher stability. Relative gene

expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The average ΔCt values for the E7 time point at 37.5 ℃ were used as the calibrator.

RNAscope in-situ hybridization procedure

Formalin-fixed paraffin-embedded tissue blocks (n = 2) were cut into 5 μm sections using a microtome (Microm HM 355S; Thermo Fisher Scientific, Waltham, MA). The sections were mounted on Superfrost-Plus glass slides (Electron Microscopy Sciences, Hatfield, PA). The RNAscope in situ hybridization procedure was performed using the RNAscope™ 2.5 HD Assay-RED according to the instructions of Advanced Cell Diagnostics (ACD). Briefly, tissue sections were baked at 60 ℃ for 1 h, followed by deparaffinization by xylene. After blocking endogenous peroxidase activity with hydrogen peroxide, tissue sections were boiled in target retrieval solution and digested with protease to increase tissue permeability and allow the custom probes to access the target RNA. Target RNA was hybridized with custom probes, and then the hybridization signals were detected using a combination of amplifiers and substrates. 50% hematoxylin Gill I (Sigma-Aldrich, St. Louis, MO) was used to counterstain the sections. Images were captured with a Nikon Eclipse 80i microscope and a Nikon DS-Ri1 camera in bright field at 200 × magnification.

Statistical analysis

Statistical analysis was performed using JMP Pro 15 (SAS Institute Inc., Cary, NC). The difference in relative gene expression within day between the 37.5 ℃ and 39.5 ℃ incubation temperatures from E13 to DOH was analyzed by t test. Significant differences between different days from E7 to DOH at only 37.5 ℃ incubation temperature were analyzed by one-way ANOVA and Tukey's test. qPCR data were normalized by log10 transformation, which was the single transformation method that best fit all of the tested genes, before significance analysis, because the data did not fit a normal distribution.

Experiment 2: mRNA profile of tight junction proteins in the chicken yolk sac

Egg incubation and tissue collection

In order to obtain fresh samples for in situ hybridization analysis and to replicate the qPCR results for YS at the optimal thermoneutral temperature, a second experiment (Experiment 2) was conducted to collect YS samples from E7 to DOH at only 37.5 ℃. Broiler eggs (n = 75) from a local hatchery were incubated at 37.5 ℃ in the basement of Litton-Reaves Hall at Virginia Tech. The room temperature was between 27.5 - 28.5 ℃, which was monitored twice daily. At embryonic day 7 (E7), E9, E11, E13, E15, E17, E19, and DOH, 6 eggs were randomly selected, embryonic chicks were euthanized by cervical dislocation, and the YS samples were collected from the eggs as previously described. Similarly, two pieces of the YS sample from each bird were fixed, embedded in paraffin and used for RNAscope in situ hybridization. Pieces of remaining YS were frozen and stored at -80 ℃ for gene expression analysis of selected tight junction proteins including ZO1, OCLN, CLDN1, JAMA, and JAM2. Primers used in Experiment 2 are the same as used in Experiment 1 (Table 5-1).

In situ hybridization, relative gene expression analysis, and statistical analysis

In situ hybridization was performed with an OCLN probe and RNAscope^{TM} 2.5 HD Assay-RED kit. Fluorescent images were captured with the same microscope and camera as described previously. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The average ΔCt values for each gene at E7 were used as the calibrator. qPCR data were also log10 transformed prior to one-way ANOVA and Tukey's test analysis using significant differences set at $P < 0.05$.

Results

Experiment 1: Effect of high incubation temperature on tight junction protein expression in the yolk sac

High incubation temperature (39.5 ℃) from E12 towards DOH affected the mRNA abundance of selected tight junction proteins and heat shock proteins compared to the normal incubation temperature (37.5 ℃) (Figure 5-1). At E13, ZO1 mRNA abundance was increased at 39.5 ℃, while OCLN and HSP90 mRNA abundance only tended to be increased at 39.5 ℃ (P = 0.08 and 0.06, respectively). There was a tendency for an increase for HSP90 at E15 (P = 0.07) at 39.5 ℃. However, there was no difference for selected TJ proteins (ZO1, OCLN, CLDN1, JAMA, and JAM2). At E17, JAMA and HSP90 mRNA abundance was greater at 39.5 ℃ than 37.5 ℃ (P < 0.05). ZO1 (P = 0.08), OCLN (P = 0.06), CLDN1 (P = 0.08), and JAM2 (P = 0.096) mRNA abundance tended to be increased at 39.5 ℃ compared to 37.5 ℃. In contrast, at E19, JAMA mRNA abundance was significantly decreased at 39.5 ℃. At DOH, OCLN mRNA was significantly lower at 39.5 ℃, while JAMA mRNA tended to be lower at 39.5 ℃ (P = 0.08). There was no change, however, for HSP90 mRNA abundance at 39.5 ℃ at E19 and DOH.

There was a significant effect of age on the mRNA abundance of all selected tight junction proteins and heat shock proteins in the YS at 37.5 ℃. All tested TJ proteins showed similar expression patterns, which were greater in the early and later incubation stages, and lower in the middle incubation stages. At 37.5 ℃, ZO1 mRNA abundance was high between E7 and E11, decreased from E11 to E13, maintained a low expression level at E15 and E17, and then increased from E17 to E19. OCLN, CLDN1 and JAMA mRNA all had a similar expression pattern of a decrease from E7 to E13, and then an increase from E13 to DOH. JAM2 mRNA abundance decreased from E7 to E13, maintained a low level from E13 to E17, increased at E19, and then

decreased again from E19 to DOH. There was also an age effect for HSP70 and HSP90 mRNA at 37.5 ℃ from E13 to DOH. HSP70 mRNA abundance increased from E13 to E19 or DOH. HSP90 mRNA was low from E13 to E17 and then increased to DOH.

The distribution of OCLN mRNA in the YS of embryonic broilers from E7 to DOH, and the effect of heat stress on the distribution of OCLN from E13 to DOH are shown in Figure 5-2. OCLN mRNA showed low expression with the signal mainly located in endodermal epithelial cells of the YS. There was no obvious effect of high incubation temperature (at 39.5 ℃) on OCLN mRNA distribution in the YS of broiler embryos.

Experiment 2: mRNA profile of tight junction protein on chicken yolk sac

The density of the OCLN mRNA signal was very low at 37.5 ℃ and 39.5 ℃ in Experiment 1, which may be due to RNA degradation of the old FFPE samples. Thus, new yolk sac samples from E7 to DOH of broiler embryos were collected, and the mRNA profile of tight junction proteins in the chicken yolk sac was investigated. The tight junction protein mRNA expression profile in the YS of embryonic chicks from E7 to DOH at 37.5 ℃ is shown in Figure 5-3. The mRNA abundance of most of the selected tight junction proteins was greater at the earlier and later stages and lower in the middle incubation stages. ZO1, OCLN, CLDN1, and JAM2 mRNA abundance were significantly downregulated from E7 to E11, and remained low from E11 to E15 or E17. There was an increase in the later stages from E17 to DOH for ZO1 and OCLN mRNA, and from E15 to E17 or from E19 to DOH for CLDN1 mRNA. There was no increase for JAM2 mRNA expression in the later incubation stages. Unlike Experiment 1, there was no significant decrease from E7 to E13 for JAMA mRNA abundance. However, JAMA mRNA abundance in the YS was significantly increased from E13 to E15 or E17, and maintained a greater expression level from E17 to DOH.

In situ hybridization co-stained with hematoxylin showed that the density of the OCLN signal was still low under bright field (images not shown). Thus, OCLN mRNA was visualized under fluorescence to enhance detection. Fluorescence of OCLN mRNA signals in the YS of broiler embryos are shown in Figure 5-4. The mRNA signals were more obvious under fluorescence. The intensity of the OCLN mRNA signal was greater in at least two replicates of the YS samples at E7, E9, and E11, while lower at E13, E15, E17, E19, and DOH.

In situ hybridization results from Experiment 2 showed that density of the OCLN mRNA staining in the early incubation stages was greater than that in the middle stages. This result was consistent with the qPCR results. However, in the later stages, the density of OCLN mRNA appeared to be similar to the middle incubation stages, which did not match the expression pattern of the qPCR results. In the qPCR result, mRNA expression of OCLN on DOH was not only significantly greater than the middle incubation stages (E13 - E15), but also greater than the early incubation stages (E7 and E9). To investigate further the reason for the difference between the qPCR and in situ hybridization results, the stability of five reference genes was analyzed (Figure 5-5). For all 5 genes there was an increase in Ct from e15/e17 to e19/DOH, which indicates that there was a decrease in mRNA abundance. It is not surprising because the YS tissue would be degrading during these last days of incubation (Wong and Uni, 2021). This would affect the calculation of relative gene expression during the late incubation stages (E19 and DOH) using the $2^{-\Delta\Delta Ct}$ method. Thus, the apparent increase in mRNA abundance of the TJ proteins during the late embryonic periods may in part be due to a decrease in the reference genes and not an increase in the TJ genes.

Discussion

Tight junction proteins are expressed in epithelial cells of the YS of embryonic chickens

The YS is an extra-embryonic membrane derived from the midgut in embryonic birds and thus it is not surprising that the YS has a similar villi structure like the gut (Wong and Uni, 2021). In our study, in situ hybridization showed that OCLN mRNA was expressed in the YS of embryonic broilers, which verified that tight junctions were present in the YS (Mobbs and McMillan, 1979). Mobbs and McMillan (1979) showed the presence of tight junctions in the *area pellucida* and *area vasculosa* of the YS via electron microscopy. They also showed that the tight junction primarily sealed the endodermal epithelial cells and maintained the integrity of the endoderm to prevent the intercellular passage of the yolk. However, they did not exactly show the location of the tight junction protein complex in the YS. In our study, ZO1, OCLN, CLDN1, JAMA and JAM2 mRNA were shown to be expressed by the YS using qPCR, which showed that tight junction proteins were likely expressed by the YS of broiler embryos. Furthermore, OCLN mRNA was detected in the *area vasculosa* of the YS by in situ hybridization. Together these results demonstrate that the YS also contains tight junction complexes.

High incubation temperature can affect mRNA expression of tight junction proteins in the YS

High incubation temperature significantly increased embryonic mortality and reduced hatchability and yolk utilization of broiler chicks (French, 2000; Reynolds, 2019; Dayan et al., 2020). Dayan et al. (2020) found that high incubation temperature altered gene expression related to lipid or oligopeptide uptake, gluconeogenesis, and thyroid hormone regulation. However, there are no recent studies regarding the effect of high incubation temperature on the tight junction protein complex in the YS. In our qPCR results, expression of selected tight junction protein mRNA was increased or tended to be increased at 39.5 ℃ from E13 to E17, but decreased at 39.5

℃ from E19 to DOH. These results indicate that high incubation temperature could alter mRNA expression of tight junction proteins in the YS of broiler embryos.

Because the structure and function of "villi" of the YS and intestine are similar, it is important to compare the effect of heat stress on the expression of tight junction proteins in the intestine of poultry with its effects on the YS. Song et al. (2014) reported that heat stress from d22 to d42 by exposure to 33 °C for 10h per day significantly decreased the jejunal protein expression level of occludin and ZO1, increased the jejunal mucosal permeability of FD4 (fluorescein isothiocyanate dextran 4 kDa), and decreased the jejunal TER value in broilers. These results suggest that heat stress may cause leaky gut by weakening the tight junction through decreasing the tight junction proteins. A study in layers by Zhang et al. (2017) also showed similar results. They reported that exposure of 40-week-old laying hens to 33 °C over 10 d or 20 d decreased the ileal expression level of occludin and ZO1 mRNA and increased serum endotoxin level. In contrast, Wang et al. (2019) found that exposure to 35 °C from d22 to d42 (8 h per d) increased mRNA expression of tight junction proteins such as CLDN1, CLDN5, E-cadherin, occludin, and ZO1 in the jejunum of broiler chickens. Uerlings et al. (2018) demonstrated that mRNA level of OCLN in the jejunum of 9-d-old broiler chicks was not affected by heat stress, while OCLN protein level in the jejunum was increased by heat stress at 24 h post-treatment. However, the abundance of OCLN protein tended to be decreased by elevated temperature at 72 h post-treatment. After a 24 h heat stress exposure, CLDN1 mRNA level tended to be increased, while there was no change in CLDN1 mRNA expression by 72 h heat stress. These results might suggest that short-term heat stress induces mRNA or protein expression of tight junction proteins in the small intestine of broilers. It is possible that the short-term heat stress induces a defense mechanism of increased tight junction protein expression in the small intestine to strengthen the tight junction barrier and repair the leaky

structure (Uerlings et al., 2018). However, in long-term heat stress, the defense mechanism in the intestine was not sufficient to maintain normal tight junction protein expression.

Heat shock proteins regulate tight junction protein mRNA expression in the YS during short-term heat stress

As heat shock proteins are responsive to heat stress, the mRNA expression levels of HSP70 and HSP90 were determined in our study. Even though HSP70 mRNA abundance was not altered by heat stress, HSP90 mRNA abundance was induced by high incubation temperature (at 39.5 ℃) during short-term exposure (E13 - E17). The mRNA abundance of selected tight junction proteins was also significantly increased or tended to be increased at 39.5 ℃ between E13 and E17. These results suggest that the increase of mRNA abundance of tight junction proteins may be related to HSP90 expression. Dokladny et al. (2006) showed that modest heat (39 °C) caused increased expression of HSP70 protein in Caco-2 intestinal epithelial cells, while exposure to a higher temperature (41 °C) led to general upregulated expression of heat shock proteins, including HSP70 and HSP90. In addition, exposure of Caco-2 cells to high temperature (39 or 41 °C) resulted in a corresponding increased expression of occludin protein, while inhibition of HSP70 prevented the compensating effect of occludin. Varasteh et al. (2015) reported that heat stress for 5 d (38-39 °C for 8 h per d) not only resulted in greater mRNA expression level of ZO1 in the jejunum or ZO1 and claudin-1 in the ileum of 15 week-old Ross broiler chickens, but also upregulated mRNA abundance of HSP70 and HSP90 in the jejunum and ileum. These results indicate that the HSPs may play a protective role in maintaining the tight junction barrier in the intestine. It is possible in the YS that a similar increased expression of HSP induced the mRNA abundance of tight junction proteins. However, long-term high incubation temperature exposure (7 or 9 d of continuous heat stress) did not significantly change HSP70 and HSP90 mRNA abundance, which might explain

the decrease of selected tight junction proteins from E19 to DOH. Long term heat stress exposure might impair the protective mechanism from the heat shock proteins.

Tight junction protein mRNA shows embryonic age-dependent expression

All selected tight junction protein mRNA showed similar expression patterns in the two experiments. The mRNA abundance was greater in the early stages (E7) but lower in the middle incubation stages (E13), which was consistent with the RNAscope in situ hybridization images. These results indicate that mRNA expression of tight junction proteins is embryonic age-dependent with lower expression in the middle incubation stages. Tight junction proteins form a physical barrier between two adjacent cells that modulates the paracellular pathway transport of macromolecules but still allow the passage of ions (Tang and Goodenough, 2003). Thus, the lower mRNA expression of selective tight junction proteins might be associated with increased passage of ions from the yolk to embryonic circulations during the middle incubation stages. However, in the later stages, qPCR results in the two experiments showed greater expression level for almost all selected tight junction proteins, which was not reflected as lower OCLN signal density in the RNAscope images. This is likely due to the declining expression of the reference genes in the yolk sac during the period where the YS is degrading.

Summary and Conclusion

Short-term exposure to high incubation temperature increased mRNA abundance of tight junction proteins and HSP90 in the YS, which may help counter leakiness of the YS endoderm. However, long-term exposure to high incubation temperature decreased mRNA abundance of tight junction proteins in the YS of embryonic broilers.

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Table 5-1. Primers used for qPCR

Gene [a]	Forward Primer sequence (5′-3′)	Reverse Primer Sequence (5′-3′)	Amplicon size (bp)	Accession number	References
ZO1	TGTAGCCACAGCAAGAGGTG	CTGGAATGGCTCCTTGTGGT	98	XM_015278975.2	(Li et al., 2015)
OCLN	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCGTCTTCTGG	240	NM 205128.1	(Shao et al., 2013)
CLDN1	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA	115	NM 001013611.2	(Shao et al., 2013)
JAMA	GAAAACCAACCCGTGGACAT	GGAAGAGCCCTTCTGGAACTT	90	EF_102433.1	(Zhang et al., 2017)
JAM2	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA	59	NM_001006257.1	(Chen et al., 2015)
HSP70	TCGGCCGCAAGTATGATGA	CGGAAGGGCCAGTGCTT	58	NM_001006685.1	(Reynolds, 2019)
HSP90	GCAGCAGCTGAAGGAATTTGA	GGAAGCTCTAAGCCCTCTTTTGT	66	NM_001109785.1	(Reynolds, 2019)
RPLP0	GCGATTGCTCCCTGTGATG	TCTCAGGTCCGAGACCAGTGT	59	NM_204987.2	(Zhang and Wong, 2019)
RPLP1	TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG	62	NM_205322.1	(Zhang and Wong, 2019)
GAPDH	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	73	NM_204305.1	(Liu et al., 2020)
ACTB	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT	78	NM_205518.1	(Liu et al., 2020)
TAF4	CCAACTTGACTGCATTAGCTGC	TCGCGTAAACTGTCTGGTTGT	146	XM_417400.6	(Mazanko et al., 2019)

[a] ZO1, Zonula occludens-1; OCLN, Occludin; CLDN1, Claudin-1; JAMA, Junctional adhesion molecule-A; JAM2, Junctional adhesion molecule-2; HSP70, Heat shock protein 70; HSP90, Heat shock protein 90; RPLP0, Ribosomal protein lateral stalk subunit P0; RPLP1, Ribosomal protein lateral stalk subunit P1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, β - actin; TAF4, TATA-box binding protein associated factor 4.

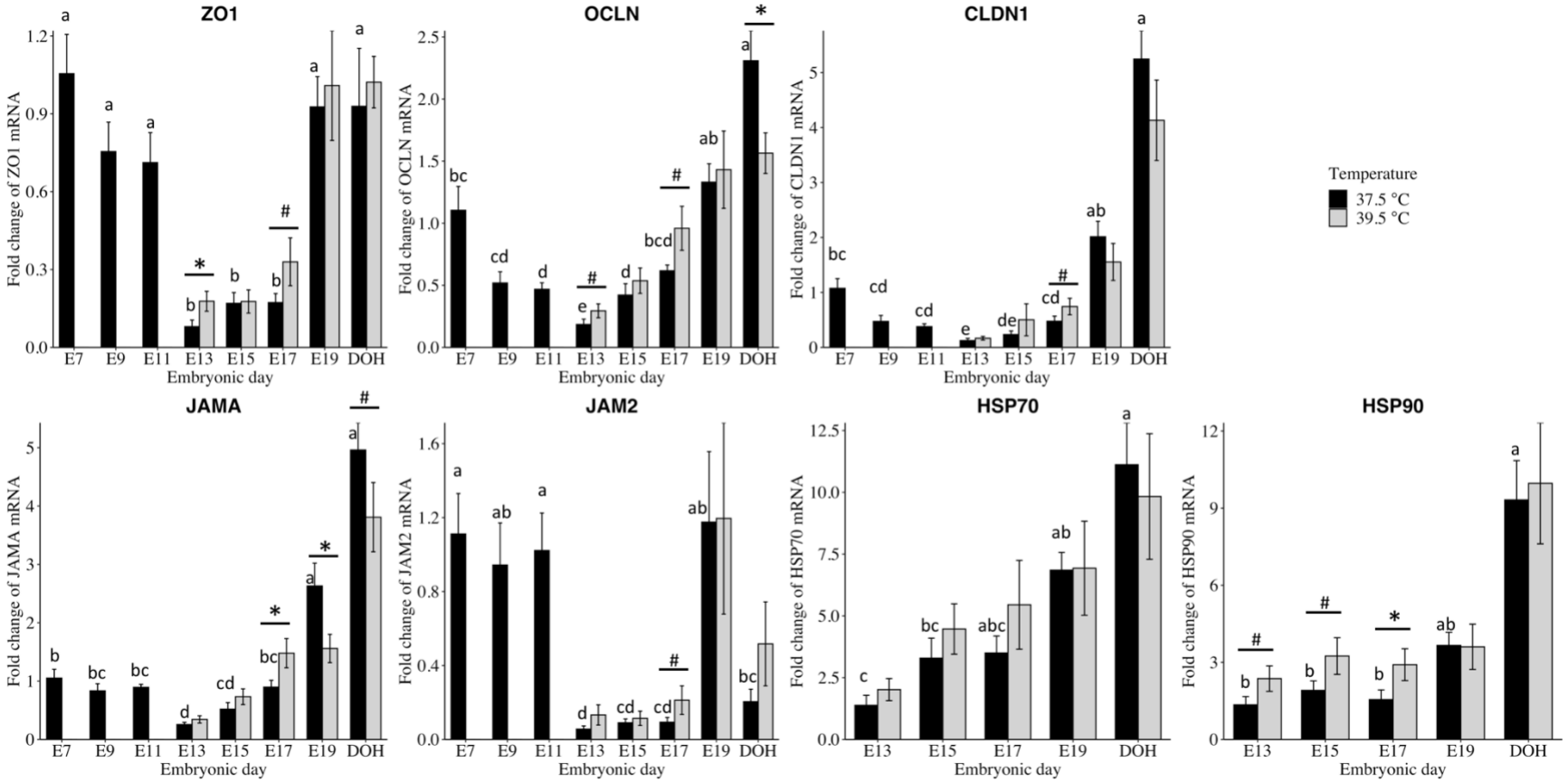

Figure 5-1: Effect of heat stress on mRNA abundance of tight junction proteins and heat shock proteins in the yolk sac of embryonic broilers. Eggs were incubated at 37.5 ℃ from embryonic day E0 to E12. At E12, 110 fertile eggs were transferred to 39.5 ℃. Yolk sac samples were collected from 6 embryonic broilers per treatment from E7 to day of hatch (DOH). Yolk sac samples (n = 6) were used for gene expression analysis using qPCR. Bars show mean ± individual SE for each gene. Different letters indicate a difference between days within 37.5 ℃ incubation temperature. * indicates difference between 37.5 ℃ and 39.5 ℃ within day ($P < 0.05$). # indicates there was a trend between 37.5 ℃ and 39.5 ℃ incubation temperature within day ($0.05 < P < 0.1$).

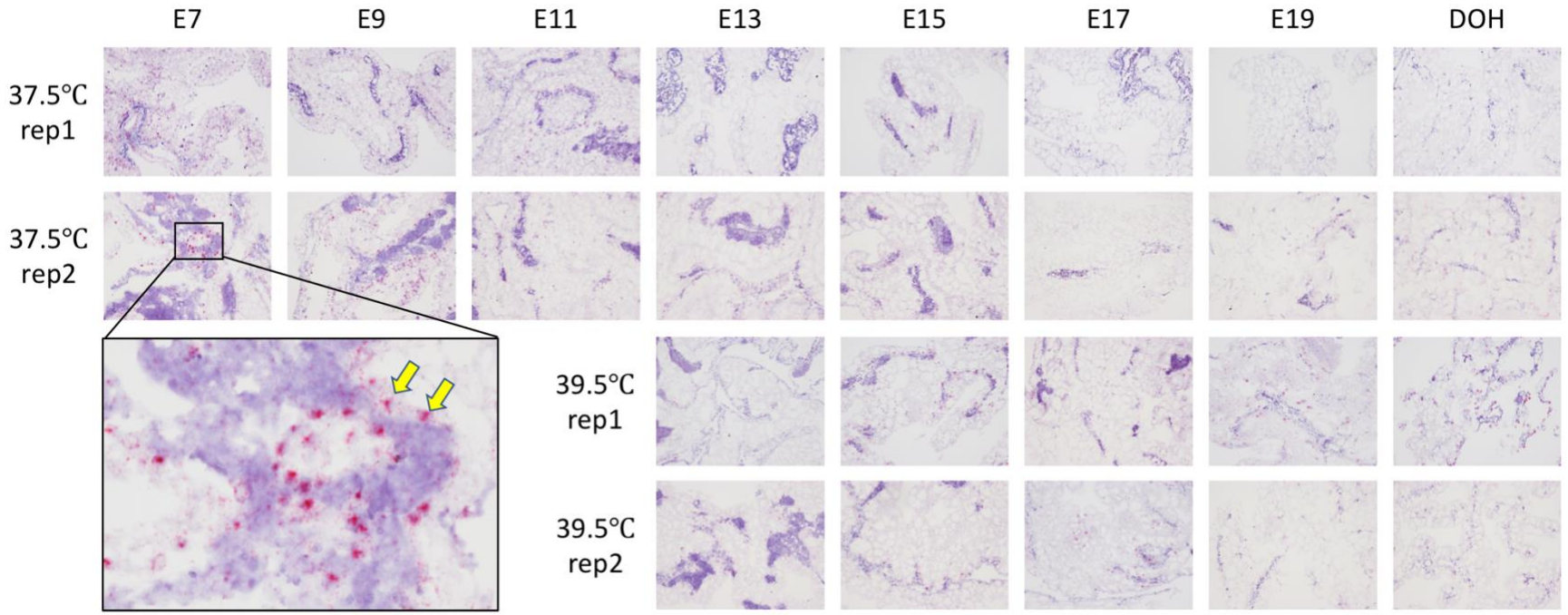

Figure 5-2: Effect of heat stress on the distribution of OCLN mRNA in the yolk sac of embryonic broilers. Yolk sac samples were collected from fertile eggs between embryonic day (E7) to day of hatch (DOH) for 37.5 ℃ and between E13 to DOH for 39.5 ℃. Formalin fixed paraffin embedded yolk sac samples were sectioned and used for in situ hybridization. OCLN mRNA (red signal) was detected using the RNAscope™ 2.5 HD Assay-RED kit. Sections were counterstained with Gill I hematoxylin. Two replicates for each temperature are shown. Images were captured at 200 × magnification. Insert shows a magnified image of an E7 sample at 37.5℃. Yellow arrows show the distribution of OCLN mRNA in the YS.

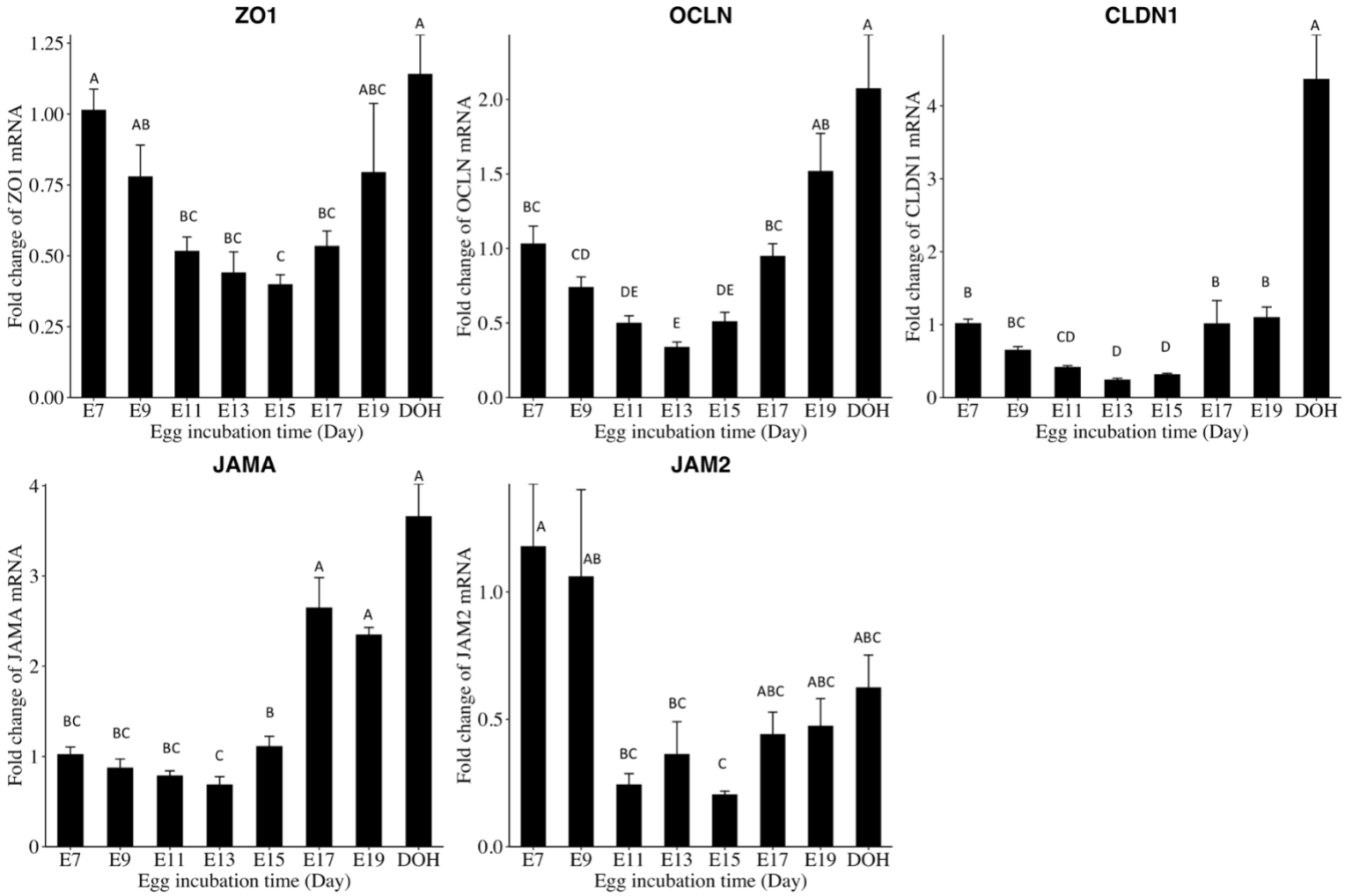

Figure 5-3: mRNA expression profile of tight junction proteins in the yolk sac of embryonic broilers from E7 to DOH. Eggs were incubated at 37.5 ℃. Yolk sac samples were collected from 6 embryonic broilers per treatment from E7 to day of hatch (DOH). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars show mean ± individual SE for each gene. Different letters indicate a difference between days for each gene.

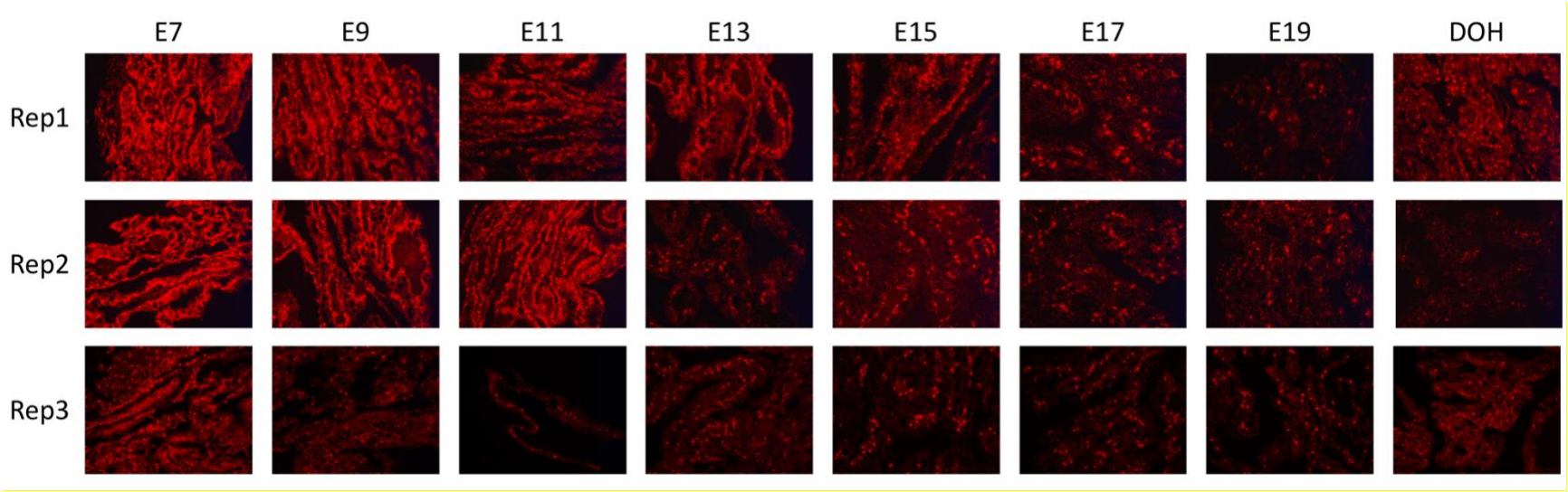

Figure 5-4: OCLN mRNA distribution in the yolk sac of embryonic broilers. Yolk sac samples were collected from embryonic day 7 (E7) to day of hatch (DOH). Formalin fixed paraffin-embedded yolk sac samples (n = 3) were processed for RNAscope in situ hybridization using an occludin probe (red). Replicate 1 (Rep1) and Rep2 were hybridized at the same time, while Rep3 was done separately.

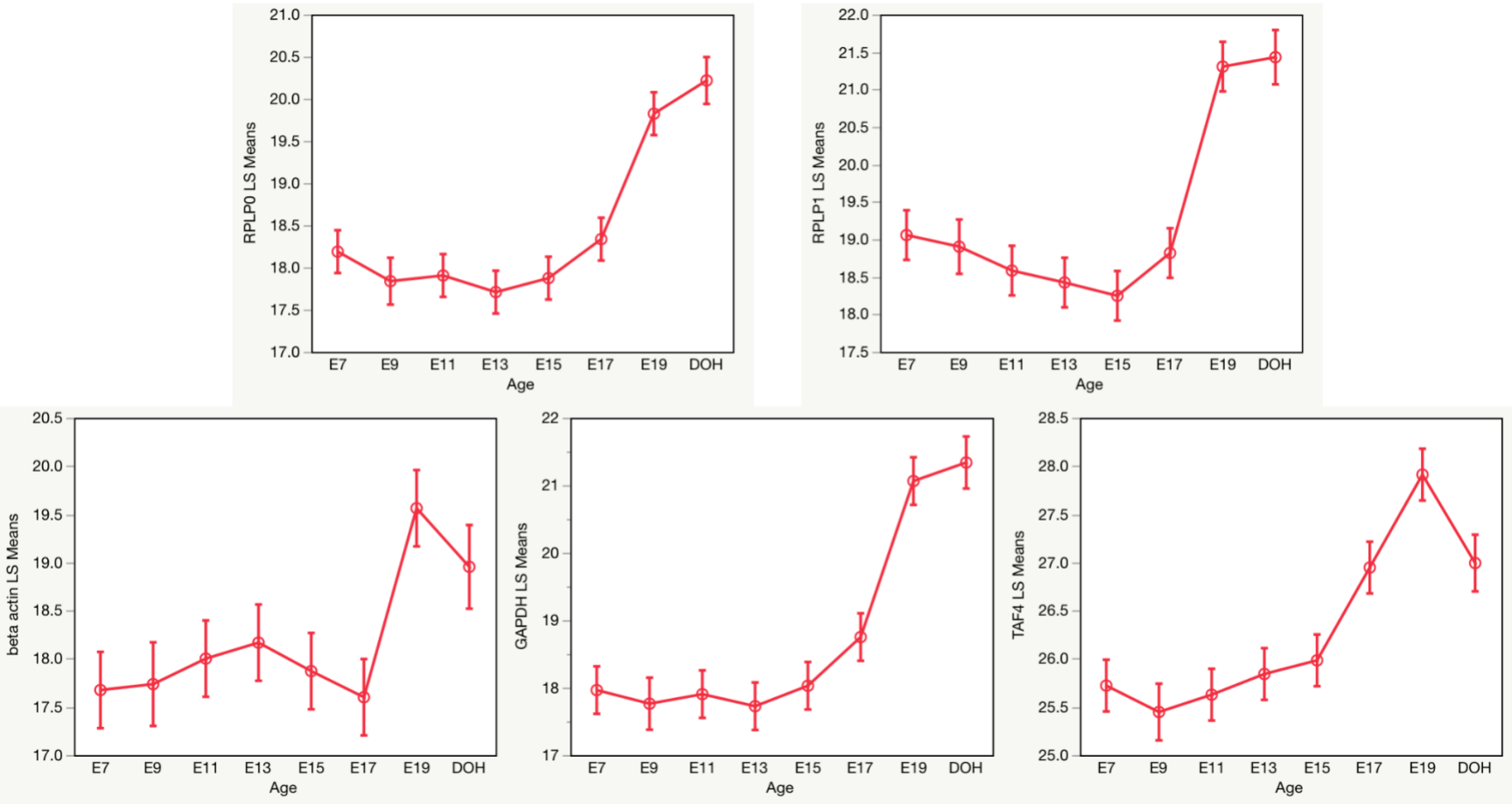

Figure 5-5: Ct values for reference genes in the yolk sac of embryonic broilers from embryonic day (E) 7 to DOH Ct value of reference genes increase from E7 to E19/DOH, indicating a decrease in mRNA abundance. RPLP0: ribosomal protein lateral stalk subunit P0; RPLP1: ribosomal protein lateral stalk subunit P1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; TAF4: TATA-box-binding protein-associated factor 4.

Chapter VI Expression of avian β defensin 10 and cathelicidins in the yolk sac of broiler and layer embryos

Abstract

The yolk sac (YS) acts as a multi-functional organ, which not only participates in nutrient absorption, but also plays an essential role in immune function by expressing defensins and by the transfer of IgY from the yolk. The objective of this study was to compare the immune function of the YS in terms of antimicrobial peptides and pathogen recognition systems in broiler and layer embryos. For experiment 1, the mRNA encoding three cathelicidins (CATHs) and three Toll-like receptors (TLRs) were investigated in the YS from embryonic day (E)7 to day of hatch (DOH). Data were analyzed by one-way ANOVA and Tukey's test. In experiment 2, mRNA abundance of avian beta defensin 10 (AvBD10) and three CATHs were compared between broilers and two layer breeds using two-way ANOVA and Tukey's test. $P < 0.05$ was considered significant for both experiments. CATH1, CATH2 and CATH3 mRNA were expressed greater in the middle incubation stages from E9 to E13, while TLR3, 4, and 7 mRNA abundance were greater at E7 or from E7 to E11. AvBD10 and CATH mRNA showed a similar expression pattern among different breeds. AvBD10 mRNA showed greater expression in layers than broilers at E9 and E11. CATH1 mRNA was generally greater in the YS of brown layers than Cobb 500 broilers. CATH2 mRNA abundance was greater in the YS of brown layers than broilers at E11. These results indicate that the YS may play an important role in the innate immune system during embryonic development by expression of antimicrobial peptides and pathogen recognition receptors. Although the temporal mRNA expression patterns for selected antimicrobial peptides were not affected by breed, their expression levels varied between breeds.

Keywords: avian beta defensin, cathelicidin, Toll-like receptor, broiler, layer

Introduction

Although eggs are considered to be a sterile environment, eggs can contain bacteria (Furuta and Maruyama, 1981) that were transferred from the hen during egg formation (Humphrey et al., 1989; Shivaprasad et al., 1990) or that had penetrated through the eggshell during oviposition and incubation (Stokes et al., 1956; Rizk et al., 1966; Cox et al., 2000). However, most fertile eggs hatch due to the presence of an innate immune system in the egg and antibodies in the yolk derived from the maternal hen (Hincke et al., 2019). The eggshell acts as a physical barrier to protect the egg content and the embryo and prevents bacterial penetration due to its cuticle (De Reu et al., 2006). The organic matrix of the eggshell also serves as a chemical barrier by expression of antimicrobial peptides such as ovocleidin-17, ovocleidin-116, and lysozyme (Mikšík et al., 2014). The egg white also has antimicrobial activity due to an alkaline environment, high viscosity, and presence of antimicrobial peptides. The alkaline pH environment is not favorable for growth of bacteria and the high viscosity of the egg white inhibits the motility of bacteria to limit the invasion rate of pathogens (Guyot et al., 2016). Antimicrobial proteins like ovotransferrin, ovalbumin, ovomucin, and lysozyme also are present in the egg white and play a potential role in innate immunity (Mine and D'Silva, 2008; Alshammari et al., 2015). Although the egg yolk provides nutrients for the embryo, it also can provide a perfect environment for bacterial proliferation. There are many antimicrobial proteins that have been identified in the egg yolk such as IgY, ovalbumin, ovotransferrin, and ovoinhibitor (Mann and Mann, 2008; Bourin et al., 2011; Zhu et al., 2020). The extra-embryonic membrane like the chorioallantoic membrane also plays an important role in defense of the egg by acting as a second physical barrier and limiting the local inflammation response (Hincke et al., 2019).

The YS is an extra-embryonic membrane that surrounds the egg yolk. The YS not only absorbs nutrients from the yolk, it also transports IgY in the yolk to the blood circulation of the embryo. This provides maternal immunity for the newly hatched chicks before their immune system matures. Zhang and Wong (2019) also found that avian β-defensin (AvBD) mRNA were expressed in the YS, which indicates that the YS also functions in innate immunity during development. AvBD1, 2, 7, and 10 mRNA in the YS were increased from the early incubation stages (E7) to the middle incubation stages (E9 to E13) followed by a decline to the later incubation stages (E19). However, AvBD1, 2, and 7 mRNA expression in the YS was different from their expression in whole embryos (Meade et al., 2009). Only the AvBD10 mRNA expression patterns matched between the YS and whole embryos. AvBD10 mRNA was greatest at E12 in the YS and whole embryos. Because the expression level of AvBD10 mRNA was the greatest relative to other AvBDs, it raises the question of what is the specific role for AvBD10.

Cathelicidins (CATHs) are another major family of antimicrobial peptides, which show antimicrobial activity against Gram-positive and Gram-negative bacteria including antibiotic resistant strains (Xiao et al., 2006). CATHs, also named fowlicidins, were also expressed in whole embryos (Meade et al., 2009). CATHB1 was not expressed at E3 and E6 and showed a low expression level at E9 and E12. CATH1, CATH2 and CATH3 had similar expression patterns with the greatest expression level at E12. However, in this study the authors did not determine CATH mRNA abundance in the YS.

Toll-like receptors (TLRs) act as a recognition system to detect pathogen associated molecular patterns (PAMPs) and initiate innate and adaptive immunity (Beutler, 2004; Iqbal et al., 2005). TLRs were also identified in embryonic tissues including the heart, liver, intestine, and brain during development (Kannaki et al., 2015). TLR4, which recognizes Gram-negative bacteria,

had the greatest expression level compared to other TLRs and the greatest levels in the liver. TLR3 and TLR7, which recognize double-stranded or single-stranded RNA, were expressed greater in the intestine. The greatest expression level for TLR3, 4, and 7 was at E12 compared with other embryonic ages from E3 to E18. Similarly, this study did not examine TLR mRNA in the YS.

Thus, one objective for this study was to investigate mRNA expression of CATHs and TLRs in the YS of broilers. The second objective was to explore the effect of different breeds including one broiler breed and two-layer breeds on the mRNA expression of AvBD10 and CATH mRNA.

Materials and methods

Experiment 1: Expression of cathelicidins and Toll-like receptors in the yolk sac

Egg incubation and tissue collection

The YS samples were from chicks housed at a thermoneutral temperature from a previous study (Reynolds, 2019). Briefly, Cobb 500 broiler eggs from a local hatchery were incubated at 37.5 ℃ at the Virginia Tech poultry farm. Six fertile eggs were randomly selected every other day from embryonic day (E) 7 to day of hatch (DOH). Embryos were euthanized by cervical dislocation before collecting samples. The YS samples were rinsed with cold 1 × phosphate-buffered saline (PBS) to remove remaining yolk. Samples were cut into small pieces, placed into tubes, frozen on dry ice, and then stored at -80 ℃ for gene expression analysis. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RNA extraction and relative gene expression analysis

An approximately 25 mg sample of the YS was homogenized in TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) using a tissue lyser. Total RNA was isolated using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). The concentration and purity of the

RNA samples were analyzed using a Nanodrop. One µg of total RNA was used to synthesize cDNA following the instructions of the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). cDNA stock was diluted 1:20 and used for real-time quantitative PCR (qPCR). Each sample was run in duplicate. Each well consisted of 5 µl SYBR green master mix (Thermo Fisher Scientific), 1 µl forward and reverse primers (5 µM), and 1.5 µl diluted cDNA sample and DEPC water. qPCR was performed using an Applied Biosystems 7500 Fast Real-time PCR system (Thermo Fisher Scientific) under the default fast program: 95 °C for 20 s, 40 cycles of 90 °C for 3 s and 60 °C for 30 s. Primers were designed by Primer Express software 3.0 (Thermo Fisher Scientific), and are listed in Table 6-1. Primers for ribosomal protein lateral stalk subunit P0 and P1 (RPLP0 and RPLP1), and ribosomal RNA (rRNA) were tested as reference genes. Reference gene stability using all samples was analyzed by RefFinder (Xie et al., 2012). Only RPLP0 and RPLP1 were selected to be used for relative gene expression analysis due to their greater expression stability. The geometric mean of the Ct value of RPLP0 and RPLP1 was used to calculate ΔCt for each sample. The calibrator used the average of ΔCt at E7 for each gene. Relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Statistical analysis

Significant differences of gene expression between days were analyzed by one-way ANOVA and Tukey's test using JMP Pro 15 (SAS Institute Inc., Cary, NC). Different letters indicate there were significant differences between days.

Experiment 2: Comparison of the expression of avian host defense peptides in the yolk sac of embryonic broilers and layers

Egg incubation and tissue collection

The expression of avian host defense peptides (AvBD10 and cathelicidins) in the YS of embryonic broilers and layers was conducted in experiment 2. Three different breeds were used including Cobb 500 broilers, a brown layer breed that was a Rhode Island Red (RIR) × White Plymouth Rock (WPR), and the white layer breed White Leghorn. Seventy five Cobb 500 eggs were obtained from a local hatchery in Harrisonburg, VA, and 75 brown layer (HYB) and 75 white layer (W36) eggs were provided by a Hy-Line hatchery (Elizabethtown, PA). Eggs were labeled and randomly placed into an OvaEasy 190 incubator (Brinsea Products Inc, Titusville, FL). The incubation temperature was set as 37.5℃. At E7, E9, E11, E13, E15, E17, E19, 6 fertile eggs from each breed were randomly selected. Embryos were euthanized by cervical dislocation and yolk-free embryos were weighed. At DOH, 6 chicks from each breed were weighed and euthanized. The YS samples were collected and rinsed with cold 1 × PBS solution. Two small pieces of the YS samples were minced on a cold sanitized metal tray, placed into cryotubes, transferred into liquid nitrogen, and stored at -80 ℃ for relative gene expression analysis. Two pieces of intact YS samples (approximately 2 cm^2 for each) were fixed in 10% neutral buffered formalin at room temperature (RT) for 24 h, dehydrated in 70% ethanol at RT for 24 h, and then transferred into fresh 70 % ethanol for shipment to StageBio (Mount Jackson, VA) for paraffin embedding. The formalin fixed paraffin embedded (FFPE) tissue was used for RNAscope in situ hybridization.

RNA isolation and relative gene expression analysis

Total RNA from the YS tissue from E7 to DOH was extracted and used for cDNA synthesis as previously described. The primers for AvBD10 and cathelicidin 1 (CATH1), CATH2, and CATH3 are listed in Table 6-1. The expression stability of the reference genes using all samples from all three breeds was tested using RefFinder (Xie et al., 2012). The five reference genes included two ribosomal proteins (RPLP0 and RPLP1), glyceraldehyde-3-phosphate

dehydrogenase (GAPDH), β -actin (ACTB), and TATA-box binding protein associated factor 4 (TAF4). The geometric mean of the Ct values of RPLP0, ACTB, and TAF4 was used to calculate ΔCt, because these were the 3 genes that showed the greatest stability. The average ΔCt for broilers at E7 was used as the calibrator. Relative gene expression of these genes in the YS during embryonic development were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

RNAscope in situ hybridization

FFPE tissue blocks were cut into 5 μm sections. RNAscope in situ hybridization was performed using an AvBD10 probe and the RNAscope™ 2.5 HD Assay-RED kit (Advanced Cell Diagnostics, Newark, CA) following the manufacturer's instructions. Briefly, tissue sections were mounted on Superfrost-Plus glass slides and baked at 60 ℃ for tissue attachment. Tissue sections on glass slides were deparaffinized with xylene, rinsed with 100 % ethanol, treated with hydrogen peroxide to block endogenous peroxidase activity, boiled in RNAscope® Target Retrieval Reagents, and incubated with protease. The AvBD10 probe was hybridized to the target mRNA. The hybridization signals were detected using the RNAscope™ 2.5 HD Assay - RED kit. Sections were counterstained with 50 % Gill I hematoxylin (Sigma-Aldrich, St. Louis, MO). Images of AvBD10 RNAscope were captured by a Nikon DS-Ri1 camera using a Nikon Eclipse 80i microscope.

Statistical analysis

The differences in weight of yolk-free embryos or chicks between breeds for each embryonic day were analyzed by one-way ANOVA and Tukey's test. Significant difference in relative gene expression between breeds and embryonic days were analyzed by 2 - way ANOVA, and multiple comparison was analyzed by Tukey's test using JMP® Pro 15 (SAS Institute Inc., Cary, NC).

Results

Expression of cathelicidin and toll-like receptor mRNAs in the yolk sac

CATH1 mRNA abundance increased from E7 to E13 and decreased from E13 to DOH (Figure 6-1). CATH2 mRNA abundance showed a decrease from E9 to DOH. CATH3 mRNA abundance had a similar expression pattern, which showed a decrease from E11 to E15 and maintained a low expression level from E15 to DOH.

TLR mRNA expression patterns were different from the CATHs (Figure 6-1). TLR3 mRNA abundance was greatest at E7, and declined from E7 to E9 though DOH. TLR4 mRNA abundance was highly expressed from E7 to E11, which decreased from E11 to E13 though E17, increased from E17 to E19, and then declined again from E19 to DOH. TLR7 had a similar expression pattern as TLR4, with the exception that there was only a non-significant increase at E19.

The increased fold change of the target genes might be due to a decreased Ct value of the target gene or increased Ct value of the reference genes. To investigate these possibilities for the increase in TLR4 and TLR7 at E19, the Ct values for TLR4, TLR7, and reference genes were plotted (Figure 6-2). The Ct value of TLR4 and TLR7 were decreased from E17 to E19, and increased again from E19 to DOH. At the same time, the Ct value of the reference gene RPLP1 was non significantly increased from E17 to E19. These results show that the increased fold change of TLR4 and TLR7 at E19 was attributed to a combination of decreased Ct value of the TLR genes and increased Ct value of the reference genes.

Comparison of the expression of avian host defense peptides in the yolk sac of embryonic broilers and layers

Weights of yolk-free embryos or chicks

To compare the rate of embryonic growth between different breeds, yolk-free embryos from E7 to E19 and chicks at DOH were weighed (Figure 6-3). At E9, E11, E13, E15, and E17, the weights of broiler embryos were greater than layers, while there was no difference between breeds from E19 to DOH. In addition, at E17, the weights of yolk-free embryos of brown layers were greater than that of white layers.

AvBD10 mRNA expression in the YS of broilers and layers

To compare the mRNA expression patterns for AvBD10 in the YS of broilers and layers, AvBD10 mRNA abundance was determined by qPCR. There was a significant interaction between breed and embryonic age for AvBD10 (Table 6-2). AvBD10 mRNA expression in the YS was not changed from E11 to E13 for embryonic broilers, while it declined from E11 to E13 for both embryonic brown layers and white layers (Figure 6-4). AvBD10 mRNA abundance was greater in the YS of embryonic layers compared to broilers at E9 and E11, however, there was no difference between the two layers at E9 but brown layers were greater than white layers at E11.

CATH mRNA expression in the YS of broilers and layers

For CATH1 mRNA there was no interaction between breed and age, but there were significant main effects of breed and age (Table 6-2). CATH1 mRNA was greater in the YS of brown layers than that of Cobb 500 broilers. CATH1 mRNA abundance increased from E7 to E9, from E9 to E11, and maintained high expression level from E11 to E19, followed by a decrease from E19 to DOH.

There was a significant interaction between breed and age for CATH2 (Table 6-2). CATH2 mRNA abundance in the YS of brown layers increased from E9 to E11, and decreased from E11 to E13 (Figure 6-4). In contrast, CATH2 mRNA did not significantly change in the YS of broilers and white layers from E9 to E11 or from E11 to E13. At E11, CATH2 mRNA abundance was

greater in the YS of brown layers than that in broiler embryos. CATH2 mRNA increased from E7 to E13 for embryonic broilers and from E7 to E11 for embryonic brown layers and white layers. There was a decline from E13 to E19 for broilers, from E13 to E17 for white layers, and from E11 to E13 and E13 to DOH for brown layers.

For CATH3 mRNA there was no interaction between breed and age, but there was a main effect of age but no main effect of breed. (Table 6-2). CATH3 mRNA increased from E7 to E9 and from E9 to E11, maintained relatively high expression levels from E11 to E17, and then declined from E17 to DOH.

Distribution of AvBD10 mRNA in the YS of broilers and layers

In order to compare the distribution of AvBD10 mRNA in both broilers and layers, RNAscope in situ hybridization was used. AvBD10 mRNA was highly expressed in the YS for all three breeds from E7 to E13, and lowly expression from E17 to DOH (Figure 6-5). These in situ hybridization results are consistent with the qPCR results from Figure 6-4. Although not quantified, there was no apparent difference between breeds at any time point.

Discussion

The YS plays an important role in the immune defense of developing embryos during incubation. It not only transports immunoglobin (IgY) from the yolk to the circulation of embryos, which provides passive immunity against pathogens, but also expresses lactotransferrin and AvBD mRNAs during incubation, which may provide direct protection for the embryos (Yadgary et al., 2014; Hincke et al., 2019; Zhang and Wong, 2019; Wong and Uni, 2021). In our previous study, Zhang and Wong (2019) showed that AvBD1, 2, 7, and 10 mRNAs were expressed in the YS with a peak expression between E9 and E13, and lower expression level at the early (E7) or later incubation stages (E19). AvBD10 mRNA had the greatest expression compared with the other

AvBDs, which raises the question of the role for AvBD10. To determine if the expression pattern of AvBD10 mRNA was the same for broilers and layers, Cobb 500 broilers, brown layers, and white layers were compared. The AvBD10 mRNA expression pattern was similar in both broilers and layers with an increase from E7 to E9 or E11, followed by a decrease from E11 to DOH. These results were consistent to the findings of Zhang and Wong (2019), which showed a common expression pattern of AvBD10. The analysis also showed that the peak of AvBD10 mRNA abundance was greater in layers compared to broilers. This suggests that the mRNA abundance for AvBD10 varies with breeds.

Breeds (broilers vs. layers) also affected embryonic development. In our study, the body weights of yolk-free embryos from E9 to E17 were lower in layers than in Cobb 500 boilers. Ohta et al. (2004) previously showed that the weights of embryos at E14 and E19 were greater in broilers than layers. Druyan (2010) reported that the relative embryonic weight (yolk-free embryonic weight/initial egg weight) was lower in layer lines (Lohmann) than that in broiler lines (Cobb and Ross) between E15 and DOH. Oxygen consumption, hematocrit, and hemoglobin levels were also lower in layers than broilers in the study by Druyan (2010). These results suggest that broilers may have different metabolic characteristics to layers, which might include the AvBD10 expression in the YS during embryo development. Due to only a set amount of resources in eggs, it is possible that broilers might utilize more energy for their growth, but less energy for immunity in the YS.

In situ hybridization for AvBD10 showed that AvBD10 mRNA abundance in the YS was affected by age, which was consistent with our qPCR results and the finding in the study of Zhang and Wong (2019). However, no apparent difference between breeds was found by in situ hybridization, which is likely related to the small fold change of AvBD10 mRNA abundance

between broilers and layers at E9 and E11. These results show that small changes in expression as measured by qPCR are difficult to observe by RNAscope in situ hybridization.

CATH mRNA expression in the YS indicates that the YS may secrete cathelicidins along with AvBDs and lactotransferrin as part of innate immunity in fertile eggs. Meade et al. (2009) reported that CATH1, CATH2 and CATH3 were expressed in whole developing embryos from E3 to E9 and in the head and abdomen at E12. CATH1, CATH2, and CATH3 protein was expressed at E3, and the mRNA abundance increased from E3 to E6, declined from E6 to E9, and increased again from E9 to E12. The expression pattern in the embryos for CATH mRNA was different from the YS. In our study, CATH mRNA peaked between E9 and E13. These results suggest that CATHs secreted by the YS may have compensated for the decline of CATHs from embryonic tissues.

The mRNA expression pattern of CATHs was similar to the mRNA expression patterns for AvBDs in this study and in the study by Zhang and Wong (2019). In this study, both AvBD10 and CATH2 mRNA abundance was greater in layers than broilers. The increased mRNA abundance for two major types of avian host defense peptides from E9 to E13 suggests that the YS may secrete antimicrobial peptides to defend against any microbes that were transferred from the maternal hen during egg formation (Ding et al., 2017; Zhang and Wong, 2019). These results indicate that layers and broilers have developmental differences including mRNA expression of antimicrobial peptides in the extra-embryonic membranes.

TLRs were also expressed in the embryos during incubation. Meade et al. (2009) showed that TLR2, TLR15 and TLR21 were expressed in the whole embryos from E3 to E12, while TLR4 was not expressed in embryos. However, Kannaki et al. (2015) reported that TLR2A, 3, 4, 5, 7, 15, and 21 mRNA were expressed in the heart, liver, intestine, and brain of chick embryos from

E3 to E18. In our study, TLR mRNA were also expressed in the YS tissue from E7 to E19. TLRs play an important role in innate immunity via recognition of pathogen associated molecular patterns (PAMPs) and initiation of production of pro-inflammatory mediators (West et al., 2006). Thus, these results indicate that both embryonic tissue and the YS have the capability to recognize PAMPs, which could trigger an immune response against any pathogens.

TLR mRNA expression was shown to be age-dependent and tissue-dependent in the embryos. TLR mRNA abundance was compared in whole embryos at E6, E9 or the head or abdomen of embryos at E12 to its respective expression at E3 (Meade et al., 2009). TLR2 mRNA abundance was upregulated at E9 and E12 relative to its expression at E3. TLR21 mRNA was increased at E6, E9, and E12 only in the embryonic head compared to E3. In contrast, TLR15 mRNA was downregulated from E3 to E6, E9, and E12 only in the head. However, TLR15 mRNA had a similar expression pattern to TLR2 and TLR21 between E6 and E12 with a trend of an increase by age. These results suggest that TLR mRNA expression in whole embryos was increased from E6 to E12. Kannaki et al. (2015) found that TLR4 was expressed the greatest among all seven TLRs by embryonic tissues from E3 to E18. They also examined the mRNA abundance of seven TLRs in embryonic heart, liver, intestine, and brain, and compared their expression at different ages. Most TLR (TLR2, TLR3, TLR4, and TLR7) mRNA were expressed greater in embryonic tissues at E12 compared to expression at E3. However, TLR15 mRNA abundance was greater at E7, while TLR5 and TLR21 mRNA were expressed greater at E18. Kannaki et al. (2015) also compared TLR mRNA abundance among tissues. Their results showed that TLR4 was highly expressed in the liver from E3 to E18, while TLR3 and TLR7 mRNA abundance was greater in the intestine during development. These results suggest that TLR mRNA abundance was affected by tissues and ages. Due to a similarity in function, the mRNA abundance of three genes (TLR3,

TLR4, and TLR7) were assessed in the YS tissue of broilers in our study. TLR mRNA was expressed greater in the YS at earlier incubation times (TLR3 at E7; TLR4 and TLR7 from E7 to E11), but decreased from E7 to E9 though DOH or from E11 to E13 though E17. These results showed that TLRs were mainly expressed in the YS at the earlier embryonic ages (E7 to E11), while expressed greater in the embryonic tissues at E12. The different expression patterns in the YS and embryonic tissue for TLR3, 4, and 7 might suggest that the capacity to recognize PAMPs developed earlier in the extra-embryonic membranes than the embryonic tissues. Together with the function of AvBDs and CATHs and their mRNA expression patterns in the YS, it is possible that exposure to bacteria derived from the hen stimulated the innate immune system in the YS via an increase of TLRs at earlier incubation stages (E7 to E11), which subsequently induced expression of antimicrobial peptides such as AvBDs and CATHs (E9 to E13). Another possibility is that mRNA expression of TLRs, AvBDs, and CATHs in the YS and embryonic tissues purely provide an innate mechanism for the embryos and newly hatched chicks, which is development-dependent (Kannaki et al., 2015).

TLR4 or TLR7 mRNA abundance were significantly increased or non-significantly increased, respectively from E17 to E19 and declined from E19 to DOH. These expression patterns were verified by checking Ct value of these two genes. Although fold change of TLR4 and TLR7 mRNA abundance in the embryonic tissues from E15 to E18 was not described, their expression patterns did not show an obvious change from E15 to E18 in the study by Kannaki et al. (2015). Thus, the change of TLR4 and TLR7 mRNA abundance in the YS at E19 may be related to other activities in the yolk sac at later stages, such as internalization of the yolk sac into the abdomen of chick embryos.

Summary and Conclusion

Embryonic broilers had greater body weight than layers. However, layers, especially brown layers, had greater mRNA abundance for antimicrobial peptides such as AvBD10, CATH1, and CATH2 in the YS, which might provide stronger innate immunity for the embryos. Both broilers and layers had similar expression patterns for AvBD10 and CATHs with a peak from E9 or E13, which suggests that the expression of host defense peptides is similar due to genetic programming. TLR3, 4, and 7 were mainly expressed during the early incubation stages at E7 or from E7 to E11.

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Table 6-1: Forward and revers primers for real time quantitative PCR.

Gene [a]	Forward Primer sequence (5′-3′)	Reverse Primer Sequence (5′-3′)	Amplicon size (bp)	Accession number
AvBD10[1]	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT	64	NM_001001609.2
CATH1	TGGCCGCTGGTCATCAG	TTCTTGATCGCCCGGTAGAG	59	NM_001001605.3
CATH2	CCGGGCGTCGATCTGA	GGTGCACTCTGTCTCCATGATG	63	NM_001024830.2
CATH3	CGATGTCACCTGCGTGGAC	TTCTCCTGATGGCTTTGTAGAGGT	122	NM_001311177.1
TLR3	ATCCATGGTGCAGGAAGTTTAAG	GGAGTCTCGACTTTGCTCAATAGC	68	NM_001011691
TLR4	TCCTCCAGGCAGCTATCAAGAT	GACAACCACAGAGCTCATGCA	74	NM_001030693.1
TLR7	ACGTCAAATGCACTGTTGTTTGT	GGGAAACCAACGTCCTGATAAC	69	NM_001011688
RPLP0[1]	GCGATTGCTCCCTGTGATG	TCTCAGGTCCGAGACCAGTGT	59	NM_204987.2
RPLP1[1]	TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG	62	NM_205322.1
GAPDH[2]	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	73	NM_204305.1
ACTB[2]	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT	78	NM_205518.1
TAF4[3]	CCAACTTGACTGCATTAGCTGC	TCGCGTAAACTGTCTGGTTGT	146	XM_417400.6

[a] AvBD10, avian β- defensin 10; CATH, cathelicidin; TLR, Toll-like receptor; RPLP0, Ribosomal protein lateral stalk subunit P0; RPLP1, Ribosomal protein lateral stalk subunit P1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, $\beta - actin$; TAF4, TATA-box binding protein associated factor 4. Primers for CATHs and TLRs were designed using Primer express 3.0. Primers for AvBD10, RPLP0, and RPLP1 were from Zhang and Wong (2019); primers for GAPDH and ACTB were from Liu et al. (2020); primers for TAF4 were from Mazanko et al.(2019).

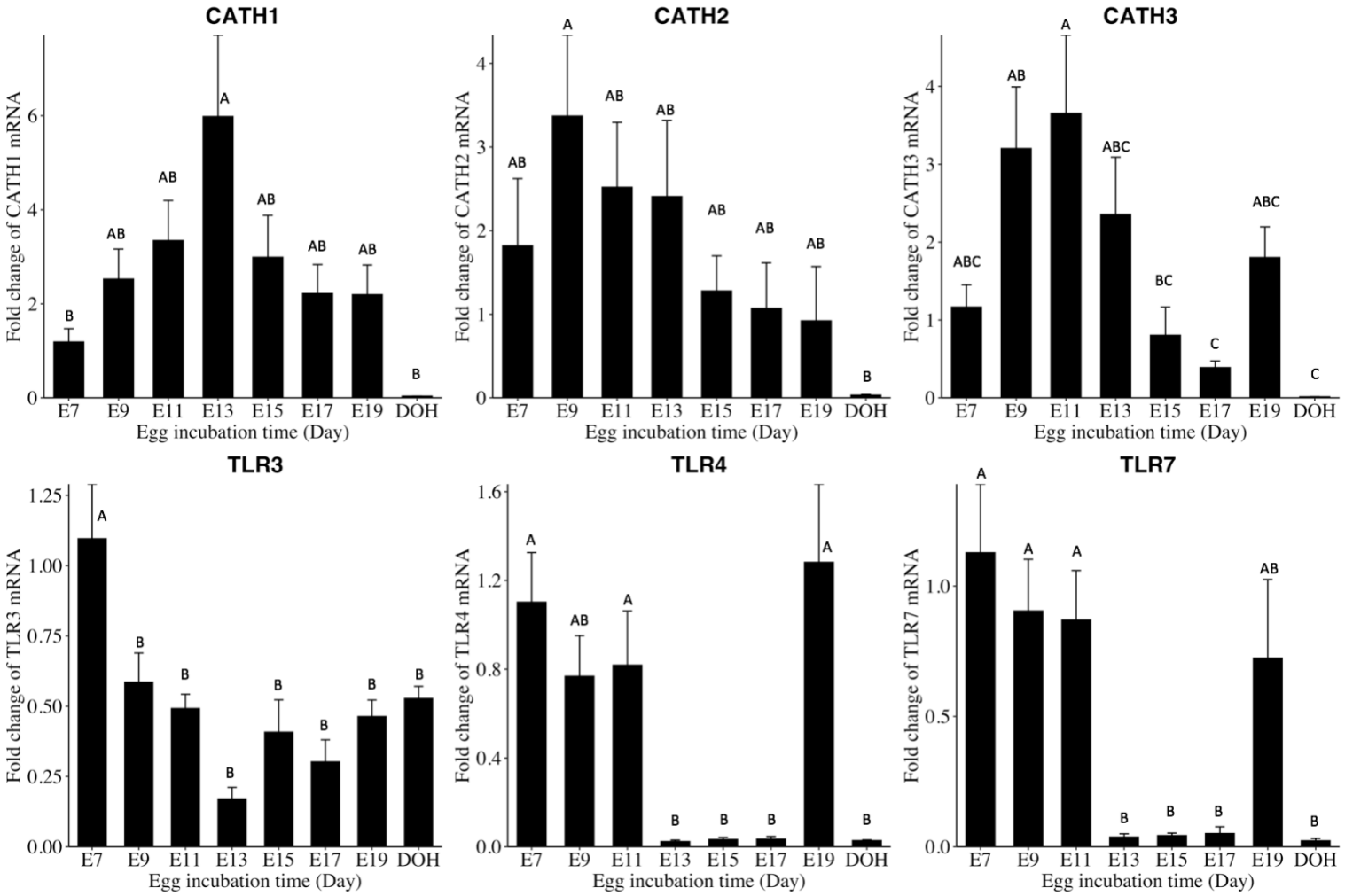

Figure 6-1: mRNA abundance of cathelicidins (CATH) and toll-like receptors (TLR) in the yolk sac of broilers from embryonic day (E) 7 to day of hatch (DOH) (n = 6). The fold change of CATH 1, 2, and 3, and TLR 3, 4, and 7 was analyzed by the $2^{-\Delta\Delta Ct}$ method. The average ΔCt at E7 for each gene was used as the calibrator. Bars show mean ± individual SE for each gene. Significant differences were analyzed by one-way ANOVA and Tukey's test. [A-C] Different letters indicate significant differences ($P < 0.05$).

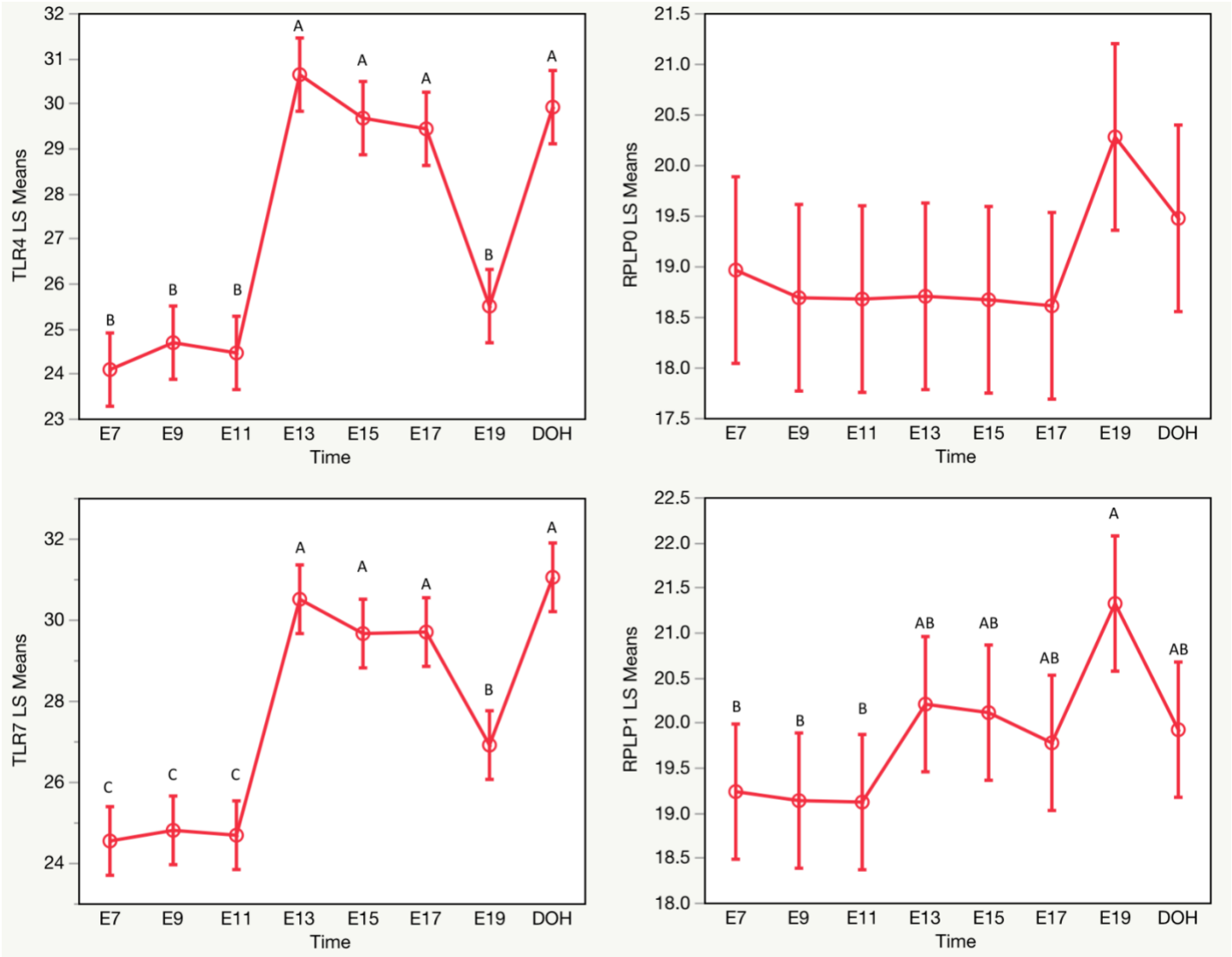

Figure 6-2: Ct value of Toll-like receptors (TLR4 and TLR7) and reference genes (RPLP0 and RPLP1) in the yolk sac of broilers from embryonic day (E) 7 to day of hatch (DOH). Significance between embryonic ages was analyzed by one-way ANOVA and Tukey's test. Different letters $^{(A\text{ - }C)}$ indicate significant differences ($P < 0.05$).

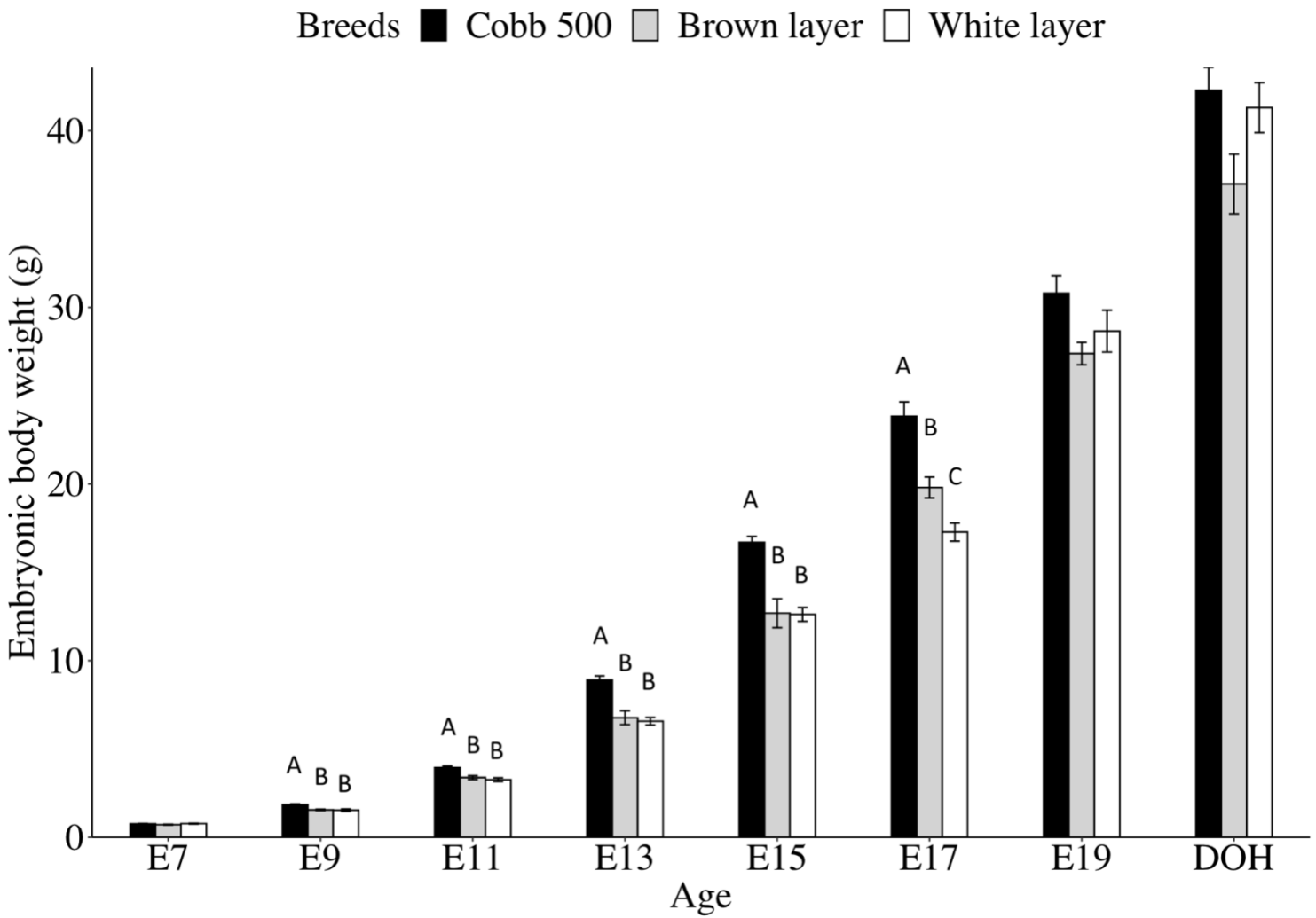

Figure 6-3: Yolk-free body weights (BW) of embryos from embryonic day (E) 7 to E19 and body weight of chicks from day of hatch (DOH) for Cobb500, brown layers and white layers. Bars show mean ± individual SE. Statistical analysis used one-way ANOVA and Tukey's test. Different letters indicate a significant difference between breeds within a time point ($P < 0.05$).

Table 6-2: Statistical analysis of fold change of avian β-defensin 10 (AvBD10) and cathelicidins (CATH 1-3) mRNA in the yolk sac of broilers and layers from embryonic day 7 (E7) to day of hatch (DOH).

	Genes[1]			
	AvBD10	CATH1	CATH2	CATH3
Breed				
Cobb 500	2.9^{c}	5.5^{b}	5.4^{b}	5.5
Brown layer	7.2^{a}	7.7^{a}	7.9^{a}	6.5
White layer	5.4^{b}	7^{ab}	7.7^{a}	5.4
SEM	0.3	0.56	0.58	0.46
P-value	$<0.0001^{*}$	0.01^{*}	0.004^{*}	0.17
Age				
E7	2.6^{c}	1.6^{c}	2.5^{ef}	1.6^{d}
E9	12.7^{a}	6^{b}	7.9^{cd}	$6.3b^{c}$
E11	11.8^{a}	10.6^{a}	14.3^{a}	9.8^{a}
E13	5.7^{b}	10.5^{a}	12.1^{ab}	8.7^{ab}
E15	3.1^{c}	7.3^{ab}	9.3^{bc}	6.6^{abc}
E17	3.2^{c}	10.3^{a}	6.1^{cde}	8.2^{abc}
E19	2^{cd}	7.5^{ab}	3.9^{def}	5.1^{c}
DOH	0.2^{d}	0.4^{c}	0.2^{f}	0.3^{d}
SEM	0.49	0.9	0.94	0.76
P-value	$<0.0001^{*}$	$<0.0001^{*}$	$<0.0001^{*}$	$<0.0001^{*}$
Interaction				
T×A	$<0.0001^{*}$	0.29	0.002^{*}	0.41

[1] Statistical analysis used two-way ANOVA and Tukey's test. Different letters indicate differences between ages or breeds (P <0.05).

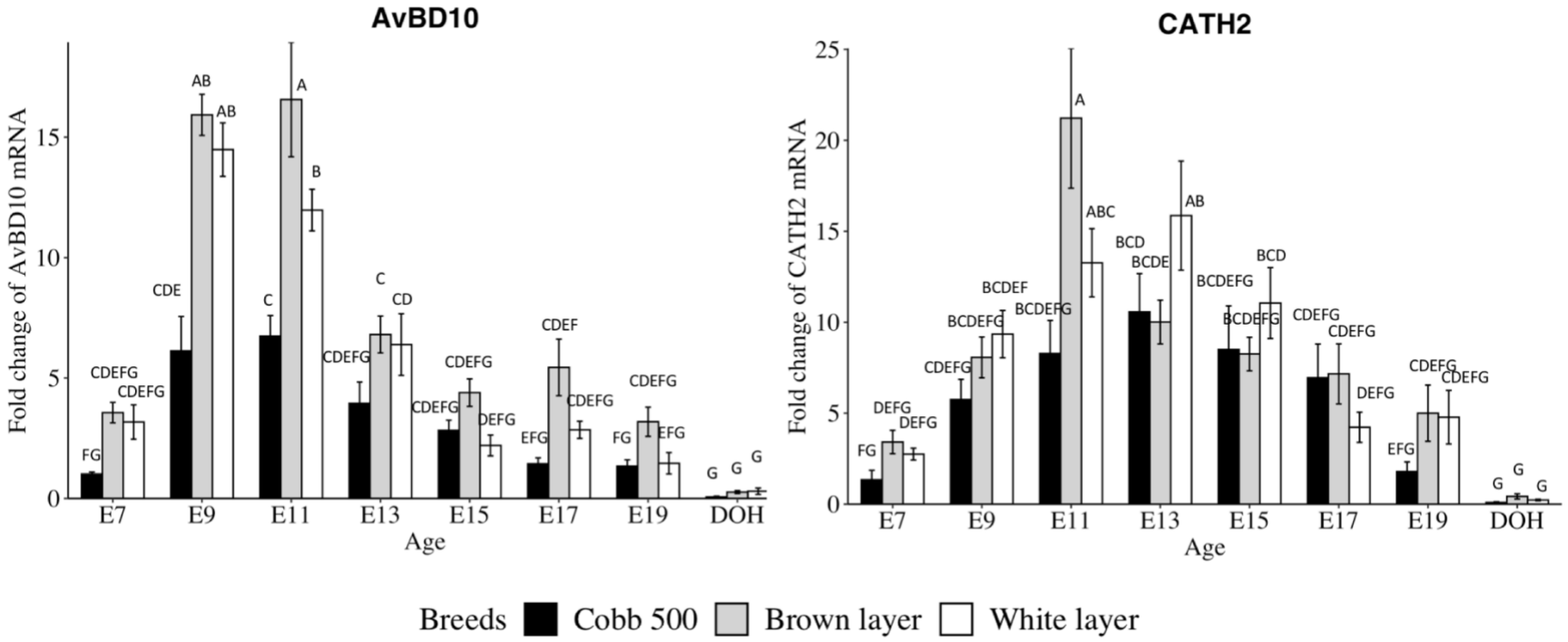

Figure 6-4: mRNA expression profiles of avian β-defensin 10 (AvBD10) and cathelicidin 2 (CATH2) in the yolk sac of broilers and layers from embryonic day 7 (E7) to day of hatch (DOH). The fold change of AvBD10 and CATH2 was analyzed by the $2^{-\Delta\Delta Ct}$ method. The average ΔCt for broilers at E7 for each gene was used as the calibrator. Bars show mean $\pm$ individual SE for each gene. Statistical analysis used two-way ANOVA and Tukey's test. Different letters indicate significant differences ($P < 0.05$).

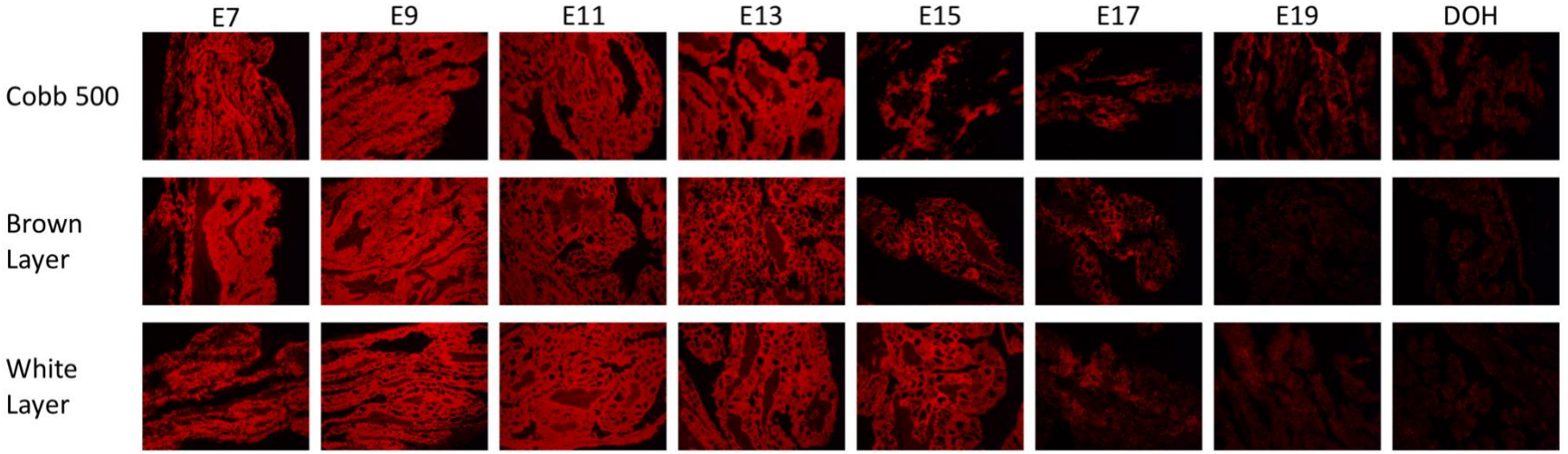

Figure 6-5: Distribution of AvBD10 mRNA in the yolk sac of broiler and layer embryos from embryonic day 7 (E7) to day of hatch (DOH). AvBD10 mRNA (red signal) was detected by in situ hybridization using the RNAscope™ 2.5 HD Assay - RED kit. Images were captured under fluorescence at 200 × magnification.

Chapter VII Epilogue

The small intestine of chickens consists of multiple types of epithelial cells. The distribution of these cells is similar to mammals. In this research, RNAscope in situ hybridization was used to identify proliferating cells in the small intestine. These cells were not only located in the epithelial cells of the crypt, but also in the blood vessels of the villi and the muscularis. These results suggest that not only intestinal stem cells, but also partially differentiated progenitor cells are proliferative, which are essential for intestinal epithelial cell homeostasis and mature cell turn-over. The tight junction protein OCLN, which maintains intestinal integrity and prevents pathogens from crossing the epithelium, is expressed only in the epithelial cells of the intestinal crypt and villus region. The effect of probiotics on the ontogeny of intestinal epithelial cells showed that probiotics did not increase marker gene expression of intestinal stem cells and goblet cells, but increased expression of a marker gene for proliferating cells and decreased expression of the antimicrobial peptide LEAP2. These results may indicate that probiotics improved cell proliferation. However, the effect of probiotics on the number of intestinal epithelial cells was not determined. In the next step, the number of different types of intestinal epithelial cells could be counted by staining using cell-specific markers. This would allow the determination of the effect of probiotics on intestinal epithelial cell numbers. The molecular mechanism of probiotics on intestinal epithelial cell proliferation would be also further investigated by detecting the expression of genes related to cell proliferation such as the Wnt signaling and Notch signaling pathways. Because the effect of probiotics on growth performance and gut health is usually bacterial strain-specific, the effect of individual strains in the probiotic products of this study could be further investigated. In this case, which specific strain promoting intestinal cell proliferation could be

determined. It is possible that only a combination of bacteria can improve cell proliferation. Next, the effect of probiotic metabolites on the ontogeny of intestinal epithelial cells could be determined, which would reveal the metabolites that promote intestinal cell proliferation. Metabolites produced by probiotics, such as lactate have been reported to improve intestinal stem cell proliferation in mice. The improvement of intestinal cell proliferation by lactate may be also shown in chickens. However, the benefits of probiotics to chickens may not only be due to metabolites, but also to the interaction between probiotics and the chicken intestine. Thus, probiotic metabolites might be only expected to show limited benefits to ontogeny of intestinal epithelial cells in chickens. Similarly, the specific strains or metabolites promoting tight junction protein expression that can improve gut integrity could also be identified. Because the tight junction not only maintains intestinal integrity, it also functions to regulate the paracellular transportation pathway for ions and water. If tight junction proteins, which function to tighten tight junctions, are too highly expressed, it is possible that the tight junction becomes too rigid, which might reduce paracellular transport.

The expression of tight junction proteins, avian defense peptides especially cathelicidins, and Toll-like receptors for pathogen recognition were profiled in the yolk sac (YS) of chicken embryos. These results suggest that the YS plays an important role in maintaining integrity of the YS and in innate immunity during the prehatch stages. My results showed that tight junction proteins were expressed in the YS with greater expression at E7 and lower expression at E13 or E15, which might indicate differences in paracellular transport of nutrients. Tight junction protein expression was affected by high incubation temperature. Transepithelial electrical resistance (TEER) assays could be utilized to show the effect of high incubation temperature on YS integrity. The regulation of heat shock proteins on tight junction protein expression in the YS could also be detected via in vitro studies. Toll-like receptors showed greater expression at E7, compared to E13

to E17; however, cathelicidins and AvBD10 expression had a peak from E9 to E13 in both broilers and layers. These results suggest that the YS plays an important role in innate immunity via expression of pathogen recognition receptors and defense peptides. Due to the development of detection technologies for bacteria, the strain and abundance of microbials can be detected, which can be correlated with expression of Toll-like receptors and antimicrobial peptides. The expression of AvBDs and CATHs could also be determined in the YS of other poultry, such as turkey, duck, or goose. In this case, the expression pattern of defense peptides in poultry and the time of peak expression during their developmental stages can be identified. This information would be helpful to explain why the peak expression of antimicrobial peptides occurs in certain developmental stages. Furthermore, the protein expression level of cathelicidins and AvBD10 could be assayed in the YS and blood vessels to determine the destination of these secreted antimicrobial peptides.