STUDY ON THE INTESTINAL CONDITIONS OF CHICKEN ORALLY ADMINISTRATED WITH *LACTOBACILLUS ACIDOPHILUS* STRAIN L-55 UNDER THE PARASITE INFECTION OR VIRUS VACCINATION

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ABSTRACT

The biggest contribution of the poultry industry is to provide meat or egg for human consumption. Annually, the industry suffers or faces losses of production due to many infectious diseases caused by bacteria, viruses, or parasites. Drugs and vaccines are the two main control measures for veterinary practices. However, due to increasing problems with prolonged drug usage and the high cost of vaccines, alternative strategies are needed for more effective and safer control of diseases in chickens. Probiotic supplement is considered as a safe alternative solution that helps to improve the gut flora, reduce inflammation, prevent and treat diarrhea or even boost the immune system. In fact, probiotic bacteria such as lactic acid bacteria (LAB) have been used in livestock production for their benefits on chick performance, immunity, and protection against enteric diseases. Nevertheless, the molecular basis of their activities, particularly related to their benefits in disease control has not been well studied. Then, I have examined the beneficial effects of LAB focused on the intestinal barrier and immune condition.

In this study*, Eimeria tenella* infection and Newcastle disease virus (NDV) vaccines were used as disease models to carry out these studies. *E. tenella* is a protozoan parasite that invades the intestinal epithelial cells in chicks, causing diarrhea and bloody stool. NDV causes Newcastle disease, which causes respiratory influenza-like symptoms in chickens, and one of the routes of invade for the virus is the intestinal tract. Therefore, it would be optimal for this research to study the usefulness of LAB to the intestinal barrier and immune responses, which is the purpose of this study. In this research, *Lactobacillus acidophilus* strain L-55 (LaL-55) was used as LAB. *L. acidophilus* is recognized as a probiotic strain with anticancer and cholesterol-lowering properties, as well as an antagonist against enteric pathogens. *L. acidophilus* has also been proven to elevate immunological activity by stimulating both the innate and adaptive immune responses. Clinical trials involving oral administration of LaL-55 showed that this strain effectively suppressed experimental allergic rhinitis and experimental atopic dermatitis in mice. In vitro studies have indicated that *L. acidophilus* is a strong inducer of T helper type 1 cytokines such as interleukin-12 and interferon (IFN)-γ. Previously, our study has indicated that LaL-55 modulated the immune reaction of chicken with an NDV attenuated vaccine.

The aims of this study are (1) to understand the relationships between clinical signs and intestinal conditions in chicken with *E. tenella*. (2) to evaluate the beneficial effect of LaL-55 under

the parasite infection or virus vaccination and its mode of action towards the intestinal integrity and the immune response.

The first experiment was conducted to investigate the relationship between *E. tenella* associated-clinical signs and molecular changes in the intestinal barrier. Chickens (1 day old) were randomly divided into three groups (15 chicks/group). The chicks in the infected group were orally inoculated with 1x 10^4 *E. tenella* oocysts at 14 days old. The level of fluorescein isothiocyanate-dextran (FITC-d) in serum was measured to evaluate the intestinal permeability. After the inoculation day, the clinical signs and number of oocysts shedding were recorded daily. Three chicks were sacrificed to collect the cecum for analyzing histopathology score and mRNA levels of junctional molecules by real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). In this study, despite the observation of diarrhea and bloody stools at 3- and 4-day post-infection (dpi), histological observations of cecum specimens showed that the disruption of the epithelial monolayer had not been occurred until 6 dpi and became more severe at 7 dpi. During the infection, the gene expressions of junctional molecules such as Claudin-3, Occludin, Zonula occludens-1, and E-cadherin were downregulated, but the gene expression of Claudin-2 was increased as compared to the control. These results may suggest that both diarrhea and bloody feces in *E. tenella* infection are related to the disruption of intestinal epithelial barrier molecules, but not a detachment of the epithelial layer. Our study is important to provide more details on the status of the chicken intestines and the expressions of intestinal barrier-related genes in response to *E. tenella* infection.

The second experiment evaluated the effects of LaL-55 probiotic administration to chicken intestine under the *E. tenella* infection model, focusing on the clinical signs and expressions of intestinal junctional genes. There were four groups in this study, including 2 control groups (with or without LaL-55) and 2 infected groups (with or without LaL-55). Chickens in the LaL-55 group were daily administrated with LaL-55 bacteria (0.75 mg/100 g body weight) from 7 days old. Chickens in the infected groups were orally inoculated with 1 x 10^4 oocysts of *E. tenella* at 14 days old. Clinical signs and the oocysts shedding were checked daily. The mRNA expression levels of Claudin-2, Claudin-3, Occludin, Zonula occludens-1, and E-cadherin were analyzed at 5 dpi by RT-qPCR. Our results indicated that, although the infected chickens administrated with LaL-55 did not show a significant difference in histopathological observations at 5 dpi, a considerably lower number of oocysts shedding was recorded as compared to that in infected chickens without

LaL-55 administration at 9 dpi. The relative mRNA levels of Claudin-2 were increased ($p < 0.01$) in both infected groups as compared to uninfected groups, however, there was no difference in the expression pattern of Claudin-2 between the two infected groups that were or were not supplemented with LaL-55. The mRNA levels of CLDN-3, OCDN, ZO-1, and E-cad among all groups showed no statistical difference, but there were tendency to be similar expression levels of these genes by LaL-55 administration in both the chicken with or without *E. tenella*.

The third experiment investigated the augmentation of chick immune responses by LaL-55 administration under NDV vaccine model. Chicks (n = 24) were randomly divided into four groups and maintained on different concentrations of LaL-55 (high-dose group, 0.5 mg/100 g body weight (BW); middle-dose group, 0.15 mg/100 g BW; low-dose group, 0.05 mg/100 g BW; and control). The NDV vaccine was inoculated at 2-week and 4-week-old chicks. We have examined gene expressions of key components of the antiviral immune responses including cytokines, melanoma differentiation-associated protein 5, and transcriptional factors at 2 weeks after the first and the second vaccination. As the virus infection is not constrained to a specific cell type, we studied the changes happening at the spleen and the ileum. For the spleen, IFN-α was significantly higher in the low and middle doses of the LaL-55 group at 6 weeks than at 4 weeks. IFN regulatory factors (IFR)-3 and IRF-7 expression was significantly higher in the low-dose group than in the middle- and high-dose groups of LaL-55. Regarding the ileum, melanoma differentiation-associated protein 5 increased in a dose-dependent manner at 4 weeks. IFN-γ and IRF-7 increased in a dose-dependent manner at 6 weeks. These results suggested that LaL-55 enhances the immune response against the NDV vaccine, albeit different mechanisms occurred between the spleen and ileum.

In conclusion, this study has evaluated the beneficial effects of LaL-55 supplementation in chicken. Our results indicate that oral administration of LaL-55 mitigates clinical signs of coccidiosis, reduces the oocyst shedding of *E. tenella* and enhances the immune response against viral infection. This information can be helpful for further researches about probiotic uses for disease prevention and control in chickens. Although LaL-55 administration did not trigger a statistically significant recovery of the gut barrier disruption by *E. tenella* infection, there was a tendency that expression levels of some barrier-related genes in infected chicks administered with LaL-55 were slightly higher than that in infected chicks without LaL-55. Future studies aimed at investigating the signaling pathways stimulated by probiotic that can regulate intestinal junctional molecules protein expression and barrier function may aid in the development of targeted therapies to prevent or reduce the severity of enteric disease in chickens.

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ANOVA:	Analysis of variance
BLIMP-1:	B-lymphocyte-induced maturation protein 1
CD4/8:	Cluster of differentiation 4/8
cDNA:	Complementary Deoxyribonucleic acid
C. perfringens:	*Clostridium perfringens*
CLDN:	Claudin
dpi:	Day post infection
E-cad:	Epithelial cadherin
Eimeria tenella:	*E. tenella*
Eimeria acervulina:	*E. acervulina*
Eimeria necatrix:	*E. necatrix*
Eimeria maxima:	*E. maxima*
Eimeria brunetti:	*E. brunetti*
Eimeria praecox:	*E. praecox*
Eimeria coli:	*E. coli*
FITC-d*:*	Fluorescein isothiocyanate dextran
h:	Hour
HE	Haematoxylin and eosin staining
IRF	Interferon regulatory factor
IL:	Interleukin
IFN-α/β/γ:	Interferon alpha/beta/gamma
Ig:	Immunoglobulin
JAM:	Junctional adhesion molecule
JAKs:	Janus kinases
L:	Litter
LAB:	Lactic acid bacteria
LaL-55:	*Lactobacillus acidophilus strain L-55*
L. acidophilus:	*Lactobacillus acidophilus*
L. reuteri:	*Lactobacillus reuteri*

L. salivarius:	*Lactobacillus salivarius*
L. plantarum:	*Lactobacillus plantarum*
L. casei:	*Lactobacillus casei*
L. rhamnosus:	*Lactobacillus rhamnosus*
L. bulgaricus:	*Lactobacillus bulgaricus*
MDA5:	Melanoma differentiation-associated protein 5
MHC:	Major histocompatibility complex
min:	Minute
MyD88:	Myeloid differentiation factor 88
mRNA:	Messenger Ribonucleic acid
µL:	MicroLit
N/No:	Number
NDV:	Newcastle disease virus
OCDN:	Occludin
PRRs:	Pattern recognition receptors
qRT-PCR:	Quantitative Reverse Transcription PCR
RPS17:	Ribosomal protein S17
SEM:	Standard error of mean
STATs:	Signal transducer and activator of transcription proteins
STING:	Stimulator of interferon genes protein
SYBR:	Synergy Brands
Temp:	Temperature
Th1/2:	T helper type 1/2
TNF-α:	Tumor necrosis factor alpha
TLRs:	Toll-like receptors
ZO:	Zonula occludins

CHAPTER I
GENERAL INTRODUCTION

1.1. Poultry industry

The most important segment of the livestock industry is the poultry sector. As an extremely complicated industry itself, the poultry sector consists of many different levels of production, including feed factories, hatcheries, breeding farms, and processing plants. Likewise, there are sub-units such as chicken, duck, turkey, and goose in terms of species in the sector. The sub-classes for production are divided into meat and egg production. Production for feathers can also be added to this. Among poultry species, chicken is the main breeding area around the world and, according to data from the United Nations Food and Agriculture Organization (FAO-2020), chicken represents more than 90 percent of the poultry sector.

The poultry sector, which has an extremely important place in terms of food safety and nutrition, is the fastest growing agricultural sub-sector, especially in developing countries. It is thought that factors such as population growth, income level growth, and urbanization will contribute to the growth of the sector in the future (Jeurissen et al., 2000). Small farms and family businesses in the sector have been instrumental in the growth of the market, it seems that the main growth will be due to large-scale operations. Although these developments contribute to the growth momentum of the market, some problems that have a negative impact also stand out as obstacles to the sector. One of these problems is infectious diseases in poultry. Besides, the risks they pose to human health due to antimicrobial resistance are also seen as a significant problem. In recent years, intensive studies have been carried out on antibiotic-free production options due to the antimicrobial resistance, therefore countries are trying to create antibiotic-free products by applying various regulations (Martin et al., 2015).

1.2. Diseases in poultry

Poultry immunity, health, and production are several factors that challenge the future growth of the poultry industry (Hafez & Attia, 2020). Poultry diseases will continue to be the primary issue for the poultry industry and its strategic future. The outbreak of any disease can turn into an epidemic and have an extensive adverse influence on the global trade of poultry products. Several factors can hasten and/or prompt the emergence of animal diseases. These factors comprise the development and structure of the poultry farming, amplify global competition and costs of production, and increase the poultry and poultry products movement worldwide. The increased movement could also raise the hazard of introducing infections to specific regions that are free

from such diseases. Resurgent and re-emerging infections are those that have occurred in the past but are now quickly growing either in a specific geographic area or in the host range. Infectious diseases and health disorders are mostly connected to negative economic impacts.

Various infectious pathogens, including bacteria, viruses, parasites, and fungi, contribute to infectious diseases in poultry and can be transmitted and subsequently spread in farms via horizontal and/or vertical transmission (Hafez, 2010). Enteric diseases, which result from infection by *E. coli*, rotavirus, coronavirus enteritis, and parasitic infestation problems are one of the most important groups of diseases that affect poultry, continuing to cause high economic losses worldwide due to increased mortality rates, decreased weight gain, increased medication costs, and increased feed conversion rates (Berkes et al., 2003). The severity of clinical signs, duration of disease, and rate of mortality and morbidity are highly variable and affected by virulence, type, and pathogenicity of the infectious agents.

1.3. The intestinal health

Intestinal health is crucial for the general health and well-being of animals and humans alike. In farm animals, feed intake and the efficient absorption of nutrients are very much determined by the health status of the gastrointestinal tract (GIT). The GIT has digestive, absorptive, metabolic, immunological and endocrinological functions. It has the most extensive exposed surface in the body and is constantly exposed to a wide variety of potentially harmful substances. The GIT acts as a selective barrier between the tissues of the chick and its luminal environment. This barrier is composed of physical, chemical, immunological, and microbiological components (Kuttappan et al., 2015). This means that disruptions of intestinal health can affect one to several systemic functions. Because of the key role of GIT in animal production, the physiological roles of the avian GIT and the consequences of possible alterations have been the focus of decades of research and extensive reviews, over the past two decades, this topic has gained even more interest in poultry production due to increasing demands for economic efficiency, animal welfare, food safety, reduction in environmental impacts, and a ban on or avoidance of growth-promoting antibiotic use (Rajput et al., 2020; Wiryawan et al., 2014).

1.4. Importance of intestinal barrier

Maintaining and improving gut health is fundamentally important as the gut supports optimal digestion and therefore performance and profitability of production. Managing gut health

through barrier function is regarded as a new frontier for disease prevention across different species (Broom & Kogut, 2018). In poultry, considerable research has been done on improving animal performance and gut health through various nutritional approaches. However, few objective measures have been identified that could relate to the functionality of the intestinal barrier and detection of inflammation. The complex structure of the epithelium, consisting of a mucus layer covering a single layer of epithelial cells, plays a crucial role in controlling the permeability and selective absorption of nutrients (Lee et al., 2018). A fundamental function of the intestinal epithelium is to act as a barrier that limits interactions between luminal contents such as the intestinal microbiota, the underlying immune system and the remainder of the body, while supporting vectorial transport of nutrients, water and waste products (Hollander, 1999). Epithelial barrier function requires a contiguous layer of cells as well as the junctions that seal the paracellular space between epithelial cells

The intestinal epithelium forms the lining of the small intestine. Each epithelium has a brush border, villi, crypt, and basolateral plasma membrane structure (Kagnoff, 2014). The small intestine not only absorbs nutrients from the diet but also offers a physical barrier assisted by the tight junctions formed by neighboring epithelial cells and a biological barrier, both of which act against harmful substances such as microorganisms, antigens, while supporting vectorial transport of nutrients, water and waste products (Hollemans et al., 2020). Epithelial barrier function requires a contiguous layer of cells as well as the junctions that seal the paracellular space between epithelial cells (Lee et al., 2018). Compromised intestinal barrier function has been associated with a number of disease states, both intestinal and systemic (Slifer & Blikslager, 2020).

1.4.1. Intestinal epithelial cells

Epithelial cells of the intestine possess three main functions. Their primary function is to absorb essential nutrients from digested foods. Secondly, because the mucosal surface of the intestine is the largest area of the body in contact with the external environment, its epithelial cells are exposed to a wide variety of foreign antigens and consequently compose a major part of the first line of defense against ingested pathogens. Finally, intestinal epithelial cells undergo a continuous cycle of cell death and regeneration, thereby eliminating cells damaged by the digestive process or harmful environmental agents (Fernando & Al-Attar, 1983).

Intestinal epithelial cells have traditionally been regarded as passive cells primarily responsible for maintaining the integrity of the intestinal barrier. However, it is now widely

appreciated that they are also important regulators of the natural and acquired immunity (Ruemmele et al., 2002). The mucosal epithelium of the digestive tract acts as a selective barrier permeable to nutritional ions and macromolecules but resisting entry of ingested pathogens and normal bacteria (Suzuki, 2013). Not only is this barrier of a physical nature, but also intestinal epithelial cells secrete chemicals inimical to bacteria, viruses, and parasites (Williams et al., 2015). For example, trefoil peptides of intestinal epithelial cells are secreted to the lumen surface of the intestine where they promote cellular repair in conjunction with mucin glycoproteins produced by goblet cells. In addition to promoting healing of epithelial monolayers after injury, Kindon et al. demonstrated that trefoil peptides and mucin glycoproteins act in a cooperative fashion to protect monolayers of intestinal epithelial cells against a variety of injurious agents (Kindon et al., 1995). Intestinal epithelial cells provide early signals important for initiation and regulation of the inflammatory response following viral, bacterial, and parasitic invasion at the intestinal surface (Artis, 2008).

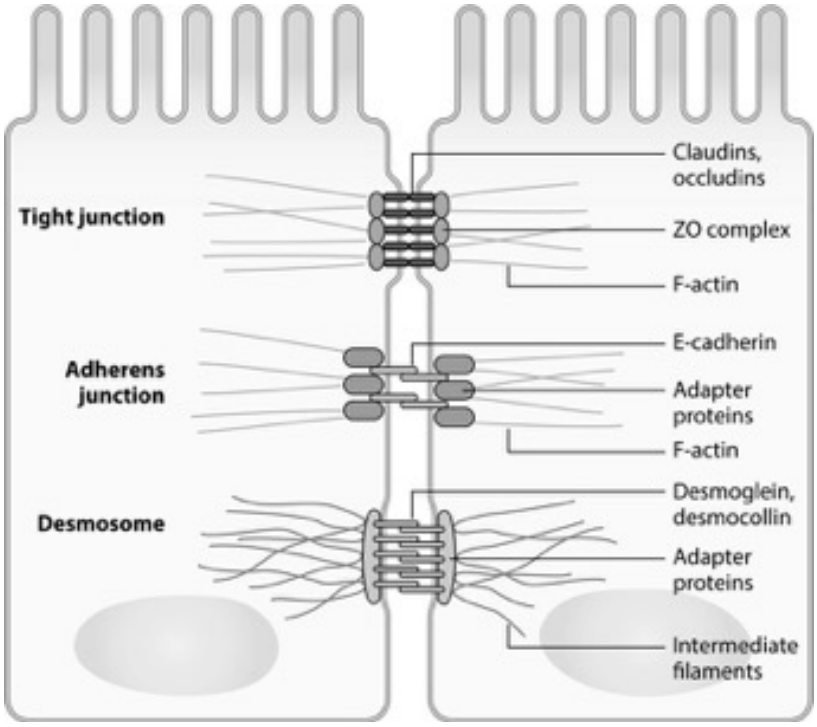
1.4.2. The apical junctional complex

In the intestinal epithelial cells, from an apical to basal direction, the intercellular junctions are the tight junction, adherens junction and desmosome (Fig. 1-1). Together these three types of intercellular junctions comprise the apical junctional complex (Furuse et al., 2001). The apical junctional complex is associated with a dense network of actin and myosin that encircles the apical aspect of each cell and supports the actin web (Sawada, 2013).

1.4.2.1. Tight junctions

Tight junctions, located at the most apical part of their lateral membranes, are cell-cell adhesion complexes that play a role in the organization of epithelial tissue. They are responsible for preserving the separation of fluid compartments that have different molecular compositions, which is of great importance for the development and maintenance of multicellular organisms (González et al., 2003). These compartments are delineated by various cellular sheets, which primarily function as barriers for maintaining their distinct internal environment. Tight junctions are predominately formed through interactions between members of the claudin family of proteins and other transmembrane components such as occludin and junctional adhesion molecules (JAMs) (Paris et al., 2008). Following the polymerization of claudin strands, the complex is strengthened by a cytoplasmic

plaque of scaffolding and adaptor proteins such as the zonula occludens (ZO) proteins (Shin and Margolis, 2006).



(Gonzalez et al., 2003)

Figure 1- 1. Apical intercellular junctions in epithelial cells. Tight junction proteins in conjunction with adherens junction proteins (cadherins, catenins). The desmosome is located basal to the adherens junction.

Claudins are a family of proteins which are the most important components of the tight junctions that provide tight control over paracellular diffusion within an epithelium. The physiological properties of tight junctions depend upon the claudin types expressed. Some claudins are classified as barrier builders, while others are classified as pore formers (Amasheh et al., 2011). For instance, claudins-1, -3, -4, -8, -11, -14 and -19 are known to decrease permeability, thus acting as 'tightening' claudins . Other claudin types mediate permeability in a charge- or ion-selective manner. Claudins-2 and -10b display cation-selectivity, whereas claudin-10a increases permeability to small anions (Suzuki, 2013). Some examples of ion-selective transport include claudin 16-mediated Ca2+ and Mg2+ reabsorption in the renal tubule and claudin 2- and 15-mediated Na+ recycling, which is essential for Na+ -dependent nutrient absorption (Na+-glucose co-transport) (Amasheh et al., 2011). Claudin 2, is largely responsible in driven decreases in epithelial barrier function are due to increases in paracellular ion conductance without accompanying alterations in flux of larger molecules. Several reporters showing that individual claudin 2-based channels are dynamically gated suggests that altering the

opening and closing of claudin 2 pores is a targetable process for barrier modulation (Shin et al., 2006; Virman et al., 2014; Yu et al., 2014)

Occludin, together with Claudins, is a tetraspanin protein normally localized to tight junctions, has been considered an important protein in regulate the formation, maintenance, and function of tight junctions (Schulzke et al., 2005). Studies have shown that rather than being important in tight junction assembly, occludin is important in tight junction stability and barrier function. An enhanced level of occludin plays a role in further improving tight junction barrier function and preventing damage to the tight junction (Kawasaki et al., 1998). On the other hand, studies in which mice were deprived of occludin expression showed morphological stability in several epithelial tissues but also found chronic inflammation and hyperplasia in the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of salivary gland, and thinning of the compact bone (Saitou et al., 2000). The phenotypical response of these mice to the lack of occludin suggests that the function of occludin is more complex than thought.

ZO proteins, comprising ZO-1, -2, and -3, are scaffolding proteins providing the structural basis for the assembly of multiprotein complexes at the cytoplasmic surface of intercellular junctions (Kagnoff, 2014). ZO connects junctional proteins such as occludin and claudin to the actin cytoskeleton, and these protein interactions maintain tight junction formation and function. Besides their structural function at cell-cell contacts, ZO proteins appear to participate in the regulation of cell growth and proliferation (Beutel et al., 2019). ZO proteins carry some domains required for structural organization of intercellular junctions and additional domains capable of functioning in signal transduction pathways. The most prominent function of ZO proteins at the junctional site is the regulation of claudin polymerization in epithelial cells, which was demonstrated by use of a reverse genetic approach (Tsukita et al., 2009). In recent years, intriguing evidence has accumulated suggesting that ZO proteins not only exert functions related to structural barrier mechanisms but are also involved in signal transduction and transcriptional modulation. In addition, ZO proteins interact directly with most of the transmembrane proteins localizing at tight junctions, such as occludin, claudins, JAM suggesting a novel function of ZO proteins, far beyond their role as indispensable structural components at the junction site (Guillemot et al., 2008; Matter & Balda, 2003).

JAM is a protein expressed at tight junctions of epithelial and endothelial cells, as well as on circulating leukocytes. Its function at tight junctions appears to be crucial as an extracellular adhesive molecule in the direct regulation of intestinal barrier function (Vetrano & Danese, 2009). JAM has been reported to influence several cellular processes, including regulation of paracellular permeability, cell polarity, cell migration, and leukocyte migration (Rehder et al., 2006).

1.4.2.2. Adherens junctions and desmosomes

Adherens junctions and desmosome are protein complexes that occur at cell-cell junctions in epithelial tissues, usually more basal than tight junctions. Adherens junctions and desmosomes provide the adhesive forces necessary for maintenance of cell-cell interactions (Guo et al., 2015). The most well-known component of the adherens junctions are the cadherins - single spanning transmembrane proteins that interact homotypically with the extracellular portion of cadherins on adjacent cells (Schneider et al., 2010). On the cytoplasmic face, cadherins interact directly with β-catenin, which in turn interact with α-catenin (Dowland et al., 2016). Among other functions, α-catenin regulates prejunctional actin assembly, which provides further strength to these structures. In addition, the adherens junction is necessary for efficient tight junction assembly, a function that in vitro studies have attributed to both epithelial cadherin (E-cadherin) and α-catenin (Ferreri and Vincent, 2008).

Desmosome is type of junctional complex, they are localized spot-like adhesions randomly arranged on the lateral sides of plasma membranes. Desmosomes are one of the stronger cell-to-cell adhesion types and are found in tissue that experiences intense mechanical stress, such as cardiac muscle tissue, bladder tissue, gastrointestinal mucosa, and epithelia (Delva et al., 2009).

1.4.3. Paracellular permeability pathways

Epithelial permeability is composed of transcellular permeability and paracellular permeability. Paracellular permeability is controlled by tight junctions. Claudins and occludin are two major transmembrane proteins in tight junctions, which directly determine the paracellular permeability to different ions or large molecules (Hu et al., 2013).

The tight junction barrier exhibits both size and charge selectivity with two distinct routes across an intact epithelial monolayer, termed the ‘pore’ and ‘leak’ pathways (Manabe et al., 2017). The pore pathway refers to a high-capacity, size-selective and charge-selective route, whereas the leak pathway is a low-capacity pathway that has more limited selectivity (Shen et al., 2011). Pore

pathway permeability seems to be determined primarily by the subset of claudins expressed, whereas leak pathway permeability can be regulated by ZO1, occludin. At sites of epithelial damage, such as erosions and ulcers, tight junctions are lost and therefore cannot contribute to local barrier function (Massier et al., 2021). Instead, luminal contents cross the intestinal barrier by a third pathway, termed the 'unrestricted' pathway (France and Turner, 2017). As its name suggests, the unrestricted pathway is high-capacity and nonselective with respect to solute size and charge. Large proteins and even whole bacteria can cross the unrestricted pathway, which partially explains the severe disease initiated by epithelial damage. In the setting of extensive epithelial injury, such as that occurring in humans with necrotizing enterocolitis or rodents treated with dextran sulfate sodium (DSS), the unrestricted pathway is often unsealed and is the predominant route of transmucosal flux (Odenwald & Turner, 2016). However, during homeostasis and less active inflammatory disease, the epithelium is generally intact and barrier function primarily reflects flux across the paracellular pore and leak pathways (Massier et al., 2021).

1.4.4. Mechanism of regulation of intestinal junctions complexes

Tight junction complexes are the rate-limiting factor for paracellular permeability. They are programmed to rapidly open and seal the barrier in the event of injury and other signals. They form a highly dynamic entity, continuously transmitting signals to the individual components that undergo a series of regulations to enhance or modulate the integrity of the intestinal barrier (Marchiando et al., 2010a).

Tight junction proteins are closely regulated, which is imperative for the maintenance of normal barrier integrity. Intestinal epithelial cells proliferate rapidly and renew quickly, and it is essential that the tight junction proteins are also strictly regulated to avoid any detrimental effect on membrane integrity(Betanzos et al., 2003). They are also capable of efficiently adapting to the different demands of the cell by sealing, opening, and maintaining paracellular transport under various physiological and pathological conditions (Harhaj and Antonetti, 2004).

The mechanism of the regulation of tight junction proteins is intricate and somewhat obscure. The tight junction proteins are regulated by multiple signaling proteins and signaling molecules. Some significant evidences have highlighted the role of cytokines in the regulation of various tight junction proteins in a multitude of pathological conditions. Tumor necrosis factor-α (TNFα), interferon-γ (IFN-γ), and interleukins all are well-known for their indisputable role in the regulation of tight junction integrity (Capaldo and Nusrat, 2009). TNFα is a key player in the

caveolin-1-mediated internalization of occludin, which elevates gut permeability; further, the overexpression of occludin alleviates the cytokine-induced increase in gut permeability (Marchiando et al., 2010b). TNFα stimulation of the nuclear factor-kappa B (NFκB) signal transduction pathway is another major mechanism involved in tight junction regulation (Wang et al., 2005). NF-κB is a central mediator of the priming signal of NLRP3 inflammasome activation and acts by inducing the transcriptional expression of NLRP3 and pro-IL-1β in response to various pattern recognition receptors ligands and cytokines (Liu et al., 2017). NF-κB inhibition protected mice from severe water loss and diarrhea, which indicated its role in the regulation of the barrier property of intestinal epithelial cells. The mechanism through which IFNγ modulates epithelial permeability is still under investigation. However, the actomyosin cytoskeletal interaction with tight junction proteins is thought to be altered by IFNγ treatment (Utech et al., 2005). IFNγ also induces an increase in barrier permeability through the reduction of ZO-1 and occludin expression in an adenosine monophosphate-activated protein kinase (AMPK)- dependent pathway, irrespective of the cellular energy levels (Rao, 2009; Scharl et al., 2009). The simultaneous presence of both these cytokines has a detrimental effect on intestinal integrity through the disassociation of tight junction proteins (Ye et al., 2006).

A prominent player in cytokine-mediated tight junction regulation is myosin light chain kinase (MLCK) (Capaldo & Nusrat, 2009; Graham et al., 2006), which disrupts the interaction between the tight junction proteins and the actin-myosin cytoskeleton, subsequently damaging the tight junction scaffold, which is crucial for the maintenance of barrier integrity (Shen, 2012; Yu et al., 2010). TNF-mediated endocytosis of the tight junction requires enhanced MLCK transcription and activity at the tight junctions. Cytokines are also responsible for occludin redistribution from the tight junction to caveolin-containing vesicles (Shen, 2012), and MLCK is also involved in the regulation of tight junction proteins through the alteration of ZO-1 protein dynamics (Cunningham & Turner, 2012).

Occludin is highly phosphorylated at serine and threonine residues in the basal epithelium (Rao, 2009; Rao et al., 2002). Phosphorylated occludin interacts with ZO-1 and other tight junction proteins. An alteration in the phosphorylation pattern, such as an increase in tyrosine phosphorylation, which results from pathological conditions, such as inflammation, can alter the protein–protein interactions of occludin with ZO-1, ZO-2, and ZO-3, and thereby alter the membrane integrity (Kale et al., 2003). Oxidative-stress-induced intestinal permeability is thought

to be mediated through the tyrosine phosphorylation of occludin and the redistribution of occludin, ZO- 1, E-cadherin, and β-catenin from the intracellular junctions (Rao, 2009). Although studies in this field have elucidated some of the mechanisms of tight junction regulation, in vivo studies that describe their role in pathological conditions are lacking.

1.4.5. Cellular functions of intestinal epithelial cells

The intestinal epithelial layer is highly dynamic and characterized by a remarkable turnover rate; intestinal epithelial cells are rapidly renewed and replaced every couple of days (Bjerknes & Cheng, 2005; Okamoto & Watanabe, 2004). The maintenance of this cell layer renewal requires tight regulation to avoid any imbalance in homeostasis (Ruemmele et al., 2002; Williams et al., 2015). The intestinal epithelial monolayer is composed of different types of specialized epithelial cells, such as enterocytes, Paneth cells, goblet cells, endocytes, and microfold cells, each with a distinct function (Artis, 2008). The most abundant of these are intestinal epithelial cells or enterocytes, for which the major function is the maintenance of epithelial barrier integrity. Paneth cells reside in the base of crypts and secrete antimicrobial peptides, such as α-defensin, to impede microbial entry to the intestinal lumen (Garcia-Hernandez et al., 2017). Goblet cells secrete mucous, trefoil peptides which are central to both the defense and repair of the epithelial layer and have significant roles in epithelial homeostasis. Endocytes regulate incoming antigens and microfold cells secrete IgA, which, in addition to goblet cells, helps present bacterial antigens to dendritic cells (Specian & Oliver, 1991). Collectively, these cells form a polarized layer to establish a tight barrier by intracellular tight junctions, adherens junctions, and desmosomes. Intestinal epithelial cells are capable of phagocytosing bacteria and can also sequester and neutralize bacterial toxins (Hu et al., 2013). These cells are also specialized to recognize bacterial-derived molecules, known as prokaryotic-associated molecular patterns, with the help of the Toll-like receptors on the cell surface and the nucleotide-binding oligomerization domain- like receptors in the cytoplasm, which activate defense mechanisms by the secretion of anti-microbial peptides (Artis, 2008). Intestinal epithelial cells also maintain two-way communication with the underlying immune cells to regulate the inflammatory response against bacterial toxins. In conjunction with the mucosal layer and specialized cells, the epithelial layer forms a well-equipped, intricately regulated and stringent barrier with continuous scrutiny by immune cells to create an immune-silent environment.

1.5. Avian coccidiosis

Coccidiosis is a big problem and one of the most important diseases of poultry worldwide. It is caused by a protozoan parasite known as *Eimeria* that invades the cells of the poultry intestine. Species of coccidia which commonly affect poultry are *Eimeria tenella, E. acervulina, E. necatrix, E. maxima and E. brunetti* (Dong et al., 2014). The disease is characterized by enteritis, diarrhea and mortality. The chicken develops reduced ability to absorb nutrients, which results in weight loss and eventually death. Subclinically, it is manifested by poor performance, impaired feed conversion, poor flock uniformity and poor growth. Coccidia can also damage the immune system and leave poultry more vulnerable to pathogens like *Clostridium*, *Salmonella* and *E. coli* (Lillehoj & Erik, 2000)

The disease is considered one of the most severe health and economical problems in poultry that causes an enormous loss to poultry producers worldwide. An outbreak of coccidiosis in a poultry flock has a very high negative and economical impact on the flock as well as for the farmer (Teng et al., 2020). There is an immediate and considerable drop in production figures and the recovery and reestablishment period after treatment is slow. Some flocks never fully recover or regain their full production potential. Hence, it is a well-recognized fact that a treatment alone cannot prevent economical losses. It is well established within the poultry sector that the only choice is therefore prevention of the disease. However, an effective and sustainable prevention and control program against the disease is not easy (Fatoba & Adeleke, 2018).

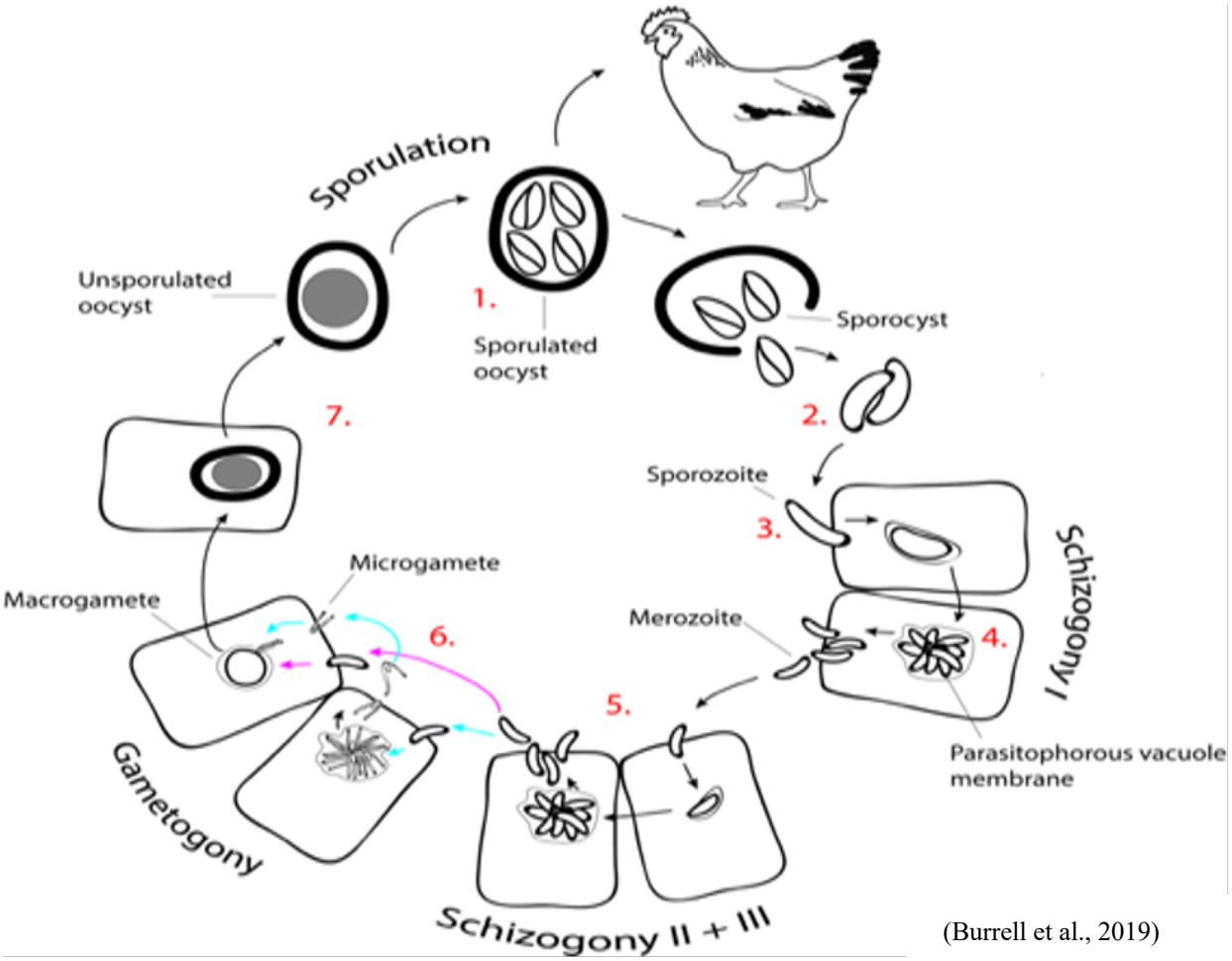
Coccidiosis is particularly difficult to combat because several different species of *Eimeria* exist in the field. Poultry may become infected with different species because the immunity that develops after infection is specific only to one species. *Eimeria* has a very complex life cycle that involves many developmental stages within the host cells. Each *Eimeria* type is able to infect only one host species and each attacks a different segment of the intestine in their host (Chapman, 2014).

The disease carries losses for the producer in the form of mortalities, reduced market value of the affected birds and sometimes culling or delayed slaughter time. Another predisposing factor is the confined host rearing conditions, which lead to an increase in the numbers of oocysts, which are ingested by poultry via the litter (Yun et al., 2000b). These lead to destruction of the integrity of the intestinal mucosa and interfere with nutrient absorption, ultimately causing diarrhea, which in turn causes high medication costs. Ultimately, all these setbacks lead to huge losses for the producer (Fatoba & Adeleke, 2018).

1.6. *Eimeria tenella* parasite

There are seven species of *Eimeria* that can infect chickens. *E. tenella*, *E. necatrix* and *E. brunetti* cause hemorrhagic coccidiosis whereas *E. acervulina, E. maxima, E. praecox,* and *E. mitis* cause malabsorptive coccidiosis (Dalloul & Lillehoj, 2006). Among them, *E. tenella* specifically infects the paired ceca in chicken and causes extensive bleeding. This acute infection occurs most commonly in young chicks and is by far the most widely diagnosed in the field due to its typical lesions (You, 2014). For this reason, a common belief is that *E. tenella* is the most prevalent all over the globe. The symptoms such as severe diarrhea and bloody stool by *E. tenella* infection are caused by the destruction of epithelial cells and small blood vessels in lamina propria, all is set upon on the complex life cycle of this parasite comprising both extracellular and intracellular stages (El-Ashram et al., 2019).

The extracellular phase of the *E. tenella* life cycle begins when unsporulated oocysts are shed into the external environment in the host feces. The oocyst sporulates by a process known as sporogony where four sporocysts each containing two sporozoites develop. Sporulated oocysts are ingested by the chicken and taken into the gizzard where the oocyst wall breaks open and sporocysts are released. Sporocysts reach the gut and on contact with the bile salts, sporozoites burst out (Lopez et al., 2020). The process by which sporozoites are released from the oocysts is known as excystation. Different *Eimeria* species are highly selective in their choice of the site of infection within chicken intestine; as mentioned above, once *E. tenella* specifically reaches the target site for infection, sporozoites invade epithelial cells of the villi tips in the caecum (Lal et al., 2009). Sporozoites then travel through the lamina propria to the crypt epithelium to undergo replication, where first-generation schizonts form. Resulting merozoites invade further crypt epithelial cells, which migrate through the basement membrane into the connective tissue, forming second-generation schizonts which cause major hemorrhagic pathology. The resulting merozoites then invade epithelial cells at the tips of the caecal folds where third-generation schizonts are formed. Third-generation merozoites then invade further epithelial cells. After three generations of merozoite production, parasite development proceeds with a single round of sexual replication known as gametogony, forming the macrogamete and microgamete. Finally, macrogamete/microgamete fertilization occurs to form a zygote. The zygote will then develop and forming an unsporulated oocyst, which releases in the feces (Burrell et al., 2019; Marugan-Hernandez et al., 2017).



(Burrell et al., 2019)

Figure 1- 2. Life cycle of Eimeria tenella. Numbers correlate with subsequent stages of the development. (1) Oocyst sporulation in the environment and oral ingestion by the chicken. (2) Release of sporocysts and sporozoites along the transit in the chicken digestive system. (3) Active invasion of sporozoites in the ceaeca epithelium and formation of the intracellular trophozoite within the parasitophorous vacuole. (4) First round of shyzogony and release of first generation merozoites. (5) Second and third rounds of shyzogony and release of second and third generation merozoites, respectively. (6) Development of microgametes and macrogametes (gametogony) and fecundation. (7) Zygote, development of the oocyst and release to the environment as unsporulated oocyst.

1.7. Intestinal immune responses to coccidiosis

1.7.1. Cell mediated immunity in coccidiosis

Cell-mediated immune responses include both antigen-specific as well as non-specific activation of T lymphocytes, NK cells, and macrophages. T lymphocytes are comprised of two functionally distinct subpopulations defined by their surface phenotypes. Cytotoxic T lymphocytes recognize foreign antigens in the context of MHC class I molecules whereas T helper cells recognize antigens in association with MHC class II molecules (Inagaki-Ohara et al., 2006).

Although cytotoxic T cells have been demonstrated in the intestine of mammals, MHC-restricted intestinal epithelial lymphocytes exhibiting cytotoxic activity have yet to be observed in chickens. In addition to MHC-restricted cytotoxic T lymphocytes, considerable interest in intestinal lymphoid populations, particularly intestinal intraepithelial lymphocytes NK cells, has developed in recent years in mammalian as well as avian immune systems (Fink et al., 2007). NK cells may constitute an active part of the first line of host cellular defense in the intestine because of their proximity to sites where antigens are introduced. The observation that chicken contained a subpopulation of NK cells that mediate spontaneous cytotoxicity indeed suggests that they are an important component of intestinal immunity (Tada et al., 2016).

The importance of T cells in acquired immunity to avian coccidia has been well documented. There is a report demonstrated a marked increase in αβ $TCR^{+}CD8^{+}$ intestinal intraepithelial lymphocytes after secondary infection with *E. acervulina* (Lillehoj, 1998). In another study, changes in intestinal T cell subpopulations in the duodenum following primary and secondary *E. acervulina* infections have been investigated and correlated with disease (Lillehoj & Erik, 2000). $CD4^{+}$ intestinal intraepithelial lymphocytes increased 7 days after primary infection in chickens and the number of $TCR^{+}CD8^{+}$ cells increased shortly after challenge infection. Following secondary infection, a significantly higher number of $CD8^{+}$ intestinal intraepithelial lymphocytes was observed in chickens, which manifested a lower level of oocyst production compared to control.

Some reports indicate that T cells are the primary effectors of immunity to *Eimeria* have led to further interest in the role of T cell subpopulations in coccidiosis (Dong et al., 2011; Kim et al., 2019). Other investigations have also characterized the dynamics of intestinal lymphocyte populations as a consequence of primary or secondary infections with *Eimeria* (Hong et al., 2006b).

1.7.2. Cytokine production during cell mediated immunity in coccidiosis

Avian cytokines have been implicated in initiating, sustaining, and regulating protective immunity against avian coccidiosis (Lillehoj et al., 2004). Of the plethora of mammalian cytokines, only a few chicken homologs have been described and the mains ones are IFN-γ, TNF, IL-1ß, IL-17, and IL-22 (Lillehoj, 1998). Among all the cytokines mentioned above, IFN-γ is a major cytokine that has anticoccidial effects (Utech et al., 2005). In mammals, parasitic infections are often characterized by increased levels of IFN-γ. Similarly, the functional role of this cytokine in Eimeria infections has been studied thoroughly (Kim et al., 2019). IFN-γ stimulates proliferation

and differentiation of hematopoietic cells and enhances non-specific immunity to tumors, bacteria, viruses, and parasites. Chicken IFN-γ regulates acquired immunity by activating lymphocytes and enhancing the expression of MHC class II antigens (Yun et al., 2000a). IFN-γ production has been used in chickens as a measure of T cell responses to coccidial antigens. Lymphocytes from Eimeria-infected chicks produced a higher level of IFN-γ compared to controls (Utech et al., 2005).

Recently, the availability of recombinant chicken IFN-γ has led to a better understanding of its physiologic and immunologic roles in chicken coccidiosis (Rothwell et al., 2010). Chicken recombinant IFN-γ was capable of protecting chick fibroblasts from virus-mediated lysis, induced nitrite secretion from macrophages in vitro, and enhanced MHC class II antigen expression on macrophages (Scharl et al., 2009). Administration of exogenous recombinant IFN-g to chickens significantly hindered intracellular development of Eimeria parasites and reduced body weight loss. When chicken fibroblast cells transfected with the IFN-g gene were infected with *E. tenella* sporozoites, significant reductions in parasite intracellular development occurred although the ability of parasites to bind and to invade host cells was not affected (Rothwell et al., 2010).

Following primary infection by *E. acervulina* or *E. maxima*, IL-17 mRNA levels were generally increased in intestinal epithelial lymphocytes when measured by a quantitative RT-PCR compared with uninfected controls (Hong et al., 2006a). The maximum increases in IL-17 expression were recorded at *E. acervulina* post-infection day 5 and day 4. In the case of *E. tenella*, IL-17 expression by gut lymphocytes following primary infection was generally down-regulated, except at day 10 post-infection. Similarly, Kim et al. (2019) reported that IL-17 expression was down-regulated in the gut following primary infection with *E. tenella*. Chickens co-infected with *E. maxima* and *Clostridium perfringens*, the etiologic agent of avian necrotic enteritis, had decreased intestinal IL-17 transcript levels compared with animals infected with *C. perfringens* alone (Park et al., 2008). Given that some studies have demonstrated that the manner of IL-17 expression in the gut is dependent on the species of infecting Eimeria as well as the time post-infection. *Eimeria* infection reduces IL-17 expression in the gut, therefore a variety of immuno-modulation strategies to increase IL-17 expression have been attempted to promote protective immunity against avian coccidiosis.

IL-22 is another cytokine produced by chickens in response to Eimeria infection. IL-22 has several roles in the gastrointestinal tract, including tissue regeneration and cell proliferation, defense against pathogens, as well as maintenance and protection of the intestinal barrier (Wang et

al., 2017). Several reporters show that IL-22 promotes epithelial wound healing as well as facilitate barrier defense mechanisms against bacterial pathogens such as *Clostridium difficile* (Zheng et al., 2008), *Citrobacter rodentium* (Zheng et al., 2008), and *Toxoplasma gondii* (Muñoz et al., 2015). T lymphocytes and macrophages are most likely the sources of cytokine production in the intestine. Intestinal lymphocytes have been observed in direct contact with parasitized epithelial cells promoting the hypothesis that they are producing cytokines and thereby modulating the immune response (Hong et al., 2006b). Concerning cytokines derived from chicken macrophages, Byrnes et al. (1993) measured the in vitro production of IL-1 and TNF-a by macrophages during and immediately following infection with *E. maxima* or *E. tenella*. Quantitatively, the amounts of IL-1 produced during each infection were nearly identical regardless of the oocyst dose. TNF-a production followed a biphasic pattern whereby the first peak was associated with disease pathogenesis and the second peak was associated with the development of protective immunity. Zhang (1995) investigated the effect of a TNF-like activity on the pathogenesis of coccidiosis in chickens. The peripheral blood leukocyte-derived macrophages produced circulating TNF-like activity in a time and dose-dependent manner.

1.8. Probiotics in poultry and their effects on gut health

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. it helps to maintain a healthy digestive system, thereby promoting the growth performance and overall health of poultry. The use of probiotics in poultry has increased steadily over the years due to the higher demand for antibiotic-free poultry and its well-researched benefits. The benefits include enhanced growth and laying performance, improved gut histomorphology, immunity, and an increase in beneficial microbiota (Wiryawan et al., 2014).

Each probiotic strain confers varying levels of protective efficacy, which is why many commercial products use multi-strain probiotics. Multiple-strain and multi-species probiotics act on different sites and provide different modes of action that create synergistic effects (Kazemi et al., 2019; Timmerman et al., 2004). The genera of probiotic microorganisms commonly used for poultry include *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Bacillus*, *Streptococcus,* and yeast such as *Candida*. The standard criteria for selecting probiotic strains include tolerance to gastrointestinal conditions, the ability to adhere to the gastrointestinal mucosa, and the competitive exclusion of pathogens (Gadde et al., 2017). Additionally, probiotics are selected based on their

survival in manufacturing, transportation, storage, application processes, and their ability to maintain viability and desirable characteristics (Yadav et al., 2016).

The mechanisms of action of probiotics are multifactorial and not fully characterized. Proposed mechanisms include the secretion of antimicrobial substances, competitive adherence to the mucosa and epithelium, the strengthening of the gut epithelial barrier, and the modulation of the immune system (Bai et al., 2013; Broom & Kogut, 2018).

1.8.1. Lactobacillus acidophilus

Lactobacillus acidophilus is a species of non-spore-bearing lactic acid bacteria and has been used as probiotics in health enhancement to improve broiler (Walter, 2008), and layer chicken performance (Hutt et al., 2006). Modes of action of probiotic Lactic acid bacteria that have been proposed include competitive exclusion toward harmful bacteria, alteration of microbial and host metabolism, stimulation of immunity (Huyghebaert et al., 2011).

1.8.2. The benefit of Lactobacillus acidophilus

1.8.2.1. Improves growth performance

The addition of either pure *Lactobacillus* cultures or mixtures of lactobacilli and other bacteria to broiler diets has been reported to improve the growth performance in chicken in many reports. Broilers supplemented with *L. acidophilus* weighed 10.1% more than birds in the control group at 42 days of age (Vantsawa et al., 2017). Dietary addition of L. acidophilus could improve intestinal health and reduce the mortality of broilers suffering from necrotic enteritis (Li et al., 2018). Olnood et al. (2015) found an improvement in body weight gain (BWG) and feed conversion ratio (FCR) of broilers fed a mixture of different *Lactobacillus* strains from 1 to 42 days of age. A consistent improvement in BWG of chickens fed a culture of Lactobacillus has also been reported (Awad et al., 2009). Feeding broiler chickens up to 6 weeks of age with a diet containing a single strain of *Lactobacillus acidophilus* or a mixture of lactobacilli significantly improved BWG and FCR. Cao et al. (2013) also found that supplementation of the broiler diets with a single strain of *Lactobacillus* (*Enterococcus faecium*) significantly improved the BW and BWG compared to the control.

Table 1- 1. Summary of the beneficial probiotic species used in poultry production.

Strain	Chracteristics	Benefits	References
Bacillus amyloliquefaciens	Root-colonizing biocontrol bacteria used to fight plant root pathogens in agriculture, aquaculture, and hydroponics.	Enhances gut health and growth performance.	(Ahmed et al., 2014)
Bacillus coagulans	Bacteria exhibits the characteristics of both genera Lactobacillus and Bacillus.	Improve growth performance and gut histomorphology.	(Zhen et al., 2018)
Bacillus licheniformis	Bacteria commonly found in soil.	Prevents necrotic enteritis and enhances growth performance.	(Cheng et al., 2017)
Bifidobacterium animalis	Bacteria found in the large intestines of most mammals.	Helps the immune system, gut physiology, and health.	(Sadeghi et al., 2015)
Bifidobacterium bifidum	Bacteria that is one of the most common probiotic bacteria that can be found in the body of mammals.	Helps the immune system and gut health.	(Pender et al., 2017)
Lactobacillus acidophilus	Bacteria found in the human and animal gastrointestinal tract and mouth.	Enhances gut health and growth performance.	(Forte et al., 2016)
Lactobacillus bulgaricus	Bacteria found in the gastrointestinal tract of mammals and naturally fermented products.	Enhances growth performance and improves immune functions.	(Cheng et al., 2017)
Lactobacillus bifermentans	Bacteria found in the human and animal gastrointestinal tract.	Enhances growth performance and digestive health.	(Timmerman et al., 2006)
Lactobacillus fermentum	Bacteria found in fermenting animal and plant materials.	Enhances growth performance, gut histomorphology, and immune functions.	(Timmerman et al., 2006)
Lactobacillus sanfranciscensis	Heterofermentative bacteria closely related or normally present in sourdough.	Enhances growth performance.	(Timmerman et al., 2006)
Lactobacillus reuteri	Bacteria that naturally inhabits the gut of mammals and birds.	Enhances growth performance, gut histomorphology, immune system, and gut health.	(Awad et al., 2010)

1.8.2.2. Improving the intestinal health

Several studies have shown that probiotics like *L. acidophilus* may help to prevent and reduce diarrhea that is associated with various diseases. Evidence on the ability of *L. acidophilus* to treat acute diarrhea in children is mixed. Some studies have shown a beneficial effect, while others have shown no effect. The addition of *L. acidophilus* to oral rehydration therapy was effective in the treatment of children with acute diarrhea by decreasing the duration of diarrhea (Simakachorn et al., 2000). Evaluating the evidence by types of acute diarrhea suggests that probiotics significantly reduced antibiotic-associated diarrhea by 52%, reduced the risk of travelers diarrhea by 8%, and that of acute diarrhea of diverse causes by 34%. Probiotics reduced the associated risk of acute diarrhea among children by 57%, and by 26% among adults (Sazawal et al., 2006). Live *L. acidophilus* reduced the incidence of radiation-induced diarrhea and the need for anti-diarrheal medication and had a significant benefit on stool consistency (Chitapanarux et al., 2010). Similarly, it may help reduce diarrhea associated with antibiotics and a common infection called *Clostridium difficile* (Vicas, 2010).

1.8.2.3. Boosting the immune system

Lactobacillus acidophilus can boost the immune system and thus help reduce the risk of viral infections. There are some studies that suggested that probiotics may prevent and improve symptoms of the common cold in humans (Kang et al., 2013; King et al., 2014). Another study carefully examined the effects of *L. acidophilus* on the gut. It found that taking it as a probiotic increased the expression of genes in the intestines involved in immune response (Van Baarlen et al., 2011). *L. acidophilus* has been proven to elevate immunological activity by stimulating both the innate and adaptive immune responses (Konstantinov et al., 2008). In vitro studies have indicated that *L. acidophilus* is a strong inducer of Th1 cytokines, including IL-12 and IFN-γ (Gackowska et al., 2006; Zeuthen et al., 2006). Also, *L. acidophilus* is involved in the enhancement of the activity of NK cells (Sunada et al., 2007). Clinical trials involving oral administration of *L. acidophilus* strain L-55 (LaL-55) show that this strain effectively suppresses experimental allergic rhinitis and experimental atopic dermatitis in mice (Sunada et al., 2008). Oral treatment of chicken with Lactobacilli including *L. acidophilus*, *L. reuteri*, and *L. salivarius* modulated systemic antibody- and cell-mediated immune responses (Brisbin et al., 2011). These results suggest that *L. acidophilus* may support a healthy immune system.

1.9. Objective

The improvement of intestinal barrier integrity by the regulation of intercellular junction protein expression or through other mechanisms has shown promising results with improvement of the disease symptoms in coccidiosis caused by *E. tenella* parasite; strategies to identify and develop novel therapeutic targets to improve gut barrier integrity have become increasingly more attractive. The improvement of gut barrier integrity alone might not be sufficient in severe inflammatory disease. However, in combination with conventional immunosuppressant drugs, such as biologic therapy approaches to improve intestinal barrier might prove beneficial.

Various research groups are also investigating the strategy of probiotic administration to improve intestinal barrier integrity. However, therapeutic intervention for the regulation of barrier integrity is an emerging topic and more investigations are essential to understand the role of intestinal barrier integrity in various diseases. The elucidation of signaling pathways involved in the regulation of the apical junction complex would allow the identification of novel barrier-restoring agents, which is imperative for deciphering novel and potent approaches for disease treatment. The aim of this study is to (1) provide a better understanding on the underlying mechanisms of the intestinal epithelial barrier in response to the negative effects of parasite infection in chicken and (2) evaluate the regulation of chicken epithelial intercellular junctions by *Lactobacillus acidophilus* in vivo and protective effects on the immunity system, to explore its probiotic potential to provide a theoretical basis and effective measures to aid in the reduction of losses from coccidiosis in the poultry industry.

CHAPTER II

THE RELATIONSHIP BETWEEN *EIMERIA TENELLA* ASSOCIATED-CLINICAL SIGNS AND MOLECULAR CHANGES IN THE INTESTINAL BARRIER

2.1. Introduction

Avian coccidiosis is a pathogenic disease in poultry that is caused by intracellular apicomplexan parasites belonging to several different *Eimeria* species closely related to human enteric pathogens, such as *Cryptosporidium* spp. (Dalloul & Lillehoj, 2006). Four *Eimeria* species, *Eimeria acervulina*, *E. necatrix, E. maxima*, and *E. tenella* are the most frequent species in the chicken (Adriana et al., 2013). Each of these parasites infects a specific place in the intestines. This protozoan parasite exhibits a complex life cycle comprising both extracellular and intracellular stages. After ingesting the sporulated oocysts (exogeneous stage of this parasite) containing the invasive form (sporozoite), sporozoites are released and invade the intestinal epithelial cells. The intracellular stage (endogenous stage) then occurs inside the host intestine's epithelial cells, which involves schizogony (asexual reproduction) followed by gametogony (sexual stage). *Eimeria tenella* specifically infects the paired ceca in chickens and causes extensive bleeding. The symptoms such as bleeding and malabsorption by *E. tenella* infection are caused by the destruction of epithelial cells and small blood vessels in lamina propria when the merozoites are released from second-generation schizonts (El-Ashram et al., 2019). Severe tissue damage occurs in the chick after parasite proliferation begins (Estela et al., 2015). When the merozoites are released from the second-generation schizont-infected epithelial cells, the parasites destroy the tissues, including micro-vessels, around the infected cells. As a result, several clinical signs such as diarrhea, bloody feces, and reduced body weight are observed, resulting in severe economic losses in the poultry industry (Burrell et al., 2019; Reid et al., 2014).

The intestinal epithelial layer forms the major barrier from the external environment and plays an essential role in food digestion and nutrient absorption. (Groschwitz & Hogan, 2009; Lechuga et al., 2017). The intestinal epithelial barrier integrity is maintained by intercellular junction molecular complexes, including tight and adherens junctions. Intestinal epithelial cells are connected strongly by tight junction proteins such as claudins (CLDNs), occludin (OCDN), and zonula occludins (ZOs) at their apical ends. These proteins are involved in paracellular pathway formation that regulates the passages of ions, solutes, and water in adjacent intercellular spaces (Hossain and Hirata, 2008; Odenwald & Tuner, 2016). Adherens junction molecules are involved in strong adhesive bonds between the epithelial cells and intercellular communications (Chida et al., 2009). Therefore, the disruption of the intestinal barrier complex is closely associated with the alterations of tight and adherens junction molecules, which affects the paracellular permeability

that contributes to gastrointestinal clinical signs such as diarrhea (Awad et al., 2017; Chow et al., 2011).

Although the clinical signs of avian coccidiosis and the life cycle of *Eimeria* have been well studied, there are a few studies conducted to investigate the relationship between the molecular basis of the gut barrier dysfunction and the *Eimeria* infection. Chen et al. (2015) has reported that increased certain cytokines and decreased OCDN induce the gut barrier failure and inflammation in jejunum mucosa of broilers, resulting in elevated levels of endotoxin and acidic glycoprotein in the chick serum. Teng et al. (2020) have shown that gene expression of CLDN-1 and Junctional adhesion molecule (JAM)-2 was linearly upregulated by challenge infection of mixed *Eimeria* spp in the jejunum at 6 days post-infection (dpi). These studies evaluated the phenomenon of attack infection against chickens administrated with three coccidia (*E. acervulina*, *E. maxima*, and *E. tenella*) mixture vaccine. Although Eimeria parasites have organ specificity, it cannot be denied that vaccination with three parasite species mixture can affect each other. Therefore, it is difficult to accurately observe the phenomenon caused by only *E. tenella* infection in the chick cecum. For this reason, our study has only focused on the *E. tenella* infection at the chick cecum. This study aims to clarify the relationship between the status of intestinal epithelial junctional molecules and the typical clinical signs of chicken infected with *E. tenella* throughout the infection's development. The relationships between clinical signs of *E. tenella* infection and the changes in gene expressions of intercellular junction molecules have been investigated throughout the time course of the infection, especially at the early stage. Besides, expression levels of pro-inflammatory cytokines that can modulate the expression of intestinal junction proteins have also been evaluated.

2.2. Materials And Methods

2.2.1. Parasite

The *E. tenella* NIAH strain which is virulent and maintained at the Laboratory of Animal Physiology in Okayama University (Okayama, Japan) was used. *E. tenella* oocysts were purified by the sugar flotation method, sporulated at 28°C in 2.5% potassium dichromate, and stored at 4°C before use.

2.2.2. Animals, tissue collection, and experimental design

Eggs (White Leghorn) were purchased from Kui poultry Co., Ltd. (Mihara, Japan). Eggs were incubated at 37.7 ± 1°C until hatching. After hatching, chicks were maintained at the

coccidian-free room, fed, and watered *ad libitum*. The chicks were housed at a constant temperature (27 ± 1°C) with a 12 h dark/light cycle. All procedures were approved by the Animal Care and Use Committee, Okayama University (OKU-2018561) and were conducted following the Policy on the Care and Use of the Laboratory Animals, Okayama University.

The chicks (n = 45) were randomly divided into three groups: Control group, chicks in this group were not treated as a control; Fasting group, chicks in this group fasted 24 h before the test as a positive control; and *E. tenella* group, chicks in this group were inoculated with mature sporulated oocysts of *E. tenella* (1×10^4 oocysts/chick) at 14 days old. Three chicks were randomly picked up and anesthetized using Pentobarbital sodium salt (Tokyo Chemical Industry. Co., Ltd., Tokyo, Japan), and sacrificed by cervical dislocation for cecum collection until 6 dpi. One of the ceca was immediately frozen at -80°C for gene expression analysis and a second one was separated three pieces (proximal, medial, and distal regions). Each of the tissue pieces was fixed with 10% formaldehyde for histopathological observation.

2.2.3. Fecal collection and oocysts counting

We sampled feces daily from 5 to 10 dpi for oocysts counting. Oocysts per gram of feces were counted by the fecal flotation method using a saturated sucrose solution (Ho et al., 2021). Briefly, fecal samples (2 g/tube) were mixed thoroughly with 10 ml of distilled water, followed by 2,500 rpm centrifuging for 5 minutes at room temperature. The supernatant then was discarded, and 10 ml of the saturated sucrose solution was added to the tubes, mixed thoroughly, and centrifuged at 2,500 rpm for 5 minutes at room temperature. The supernatant was transferred to another 15 ml centrifuge tubes and mixed well. The supernatant (10 µl) was dropped on the slide glass, covered with cover glass, and the oocysts were counted using light microscopy (triplicate/tube).

2.2.4. Histopathological observation

The middle part of formaldehyde-fixed ceca was removed and embedded in paraffin, sectioned at 6 µm thickness, and de-paraffinized. We stained the sectioned specimens using a hematoxylin-eosin (HE) solution. HE specimens (6 specimens/chick, 200 µm interval) were observed under the light microscope (Olympus FSX100, Olympus, Tokyo, Japan) to evaluate the histological score (magnification, × 200). The inflammation levels of ceca were evaluated using a 0 to +4 scoring system described previously (Table 2-1; Erben et al., 2014).

Table 2- 1. The scoring system for evaluating inflammation level.

Inflammatory cell infiltration		Epithelial changes	Mucosal architecture	Score
Severity	**Extent**			
Minimal	Mucosa	Minimal Hyperplasia		1
Mild	Mucosa and submucosa	Mild Hyperplasia		2
Moderate	Mucosa, submucosa, sometimes transmural	Moderate Hyperplasia		3
Marked	Mucosa, submucosa, often transmural	Marked Hyperplasia	Ulceration, Crypt loss	4

2.2.5. Measurement of intestinal permeability

To evaluate the permeability levels in the intestine, the plasma level of fluorescein isothiocyanate-dextran (FITC-d; MW 4,000 Da; Sigma-Aldrich Co., St. Louis, MO) was determined as described by Kuttappan et al. (2015). Briefly, FITC-d was orally administrated to chicks (from day 2^{nd} to 6^{th} dpi) in all groups. One hundred fifty minutes later, peripheral blood samples were collected from a cardiac puncture in each chick used 0.2 ml anticoagulant (Heparin sodium injection 10,000U/10 ml, AY Pharmaceuticals Co., Ltd., Tokyo, Japan) /chick, mix the sample by inverting the tube 3-4 times and allowed it to clot under room temperature for 180 minutes. Then, we spun the collected blood at 1000 × g for 15 minutes at room temperature to separate the plasma. The fluorescence intensity of FITC-d in plasma was determined with an excitation of 485 nm and an emission wavelength of 528 nm by Multimode Microplate Reader (SH-9000 serial, Corona Electric Co., Ltd., Ibaraki, Japan). The samples' fluorescence levels were converted to respective FITC-d microgram per milliliter of plasma. We made such a calculation based on a standard curve previously obtained from known levels of FITC-d.

2.2.6. Gene expression analysis

Total RNAs were extracted from ceca tissues using the RNAzol® RT Reagent (COSMO BIO Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The concentration of

RNA was quantified using a Smart Spec plus Spectrophotometer (Bio-Rad Laboratories Inc., Tokyo, Japan). One microgram of total RNA was subjected to reverse transcription with oligo (dT_{18}) primers using the ReverTra Ace® Master Mix kit (Toyobo CO., LTD, Osaka, Japan) according to the manufacturer's instructions. Following the QPCR Master Mix kit instructions, we diluted all the cDNA products 50 times with nuclear-free water and stored them at −20°C until use.

Table 2- 2. The sequence of primer pairs used for amplification of target genes.

Gene Name	Primer sequence (5' to 3')		Accession No.
	Forward	Reverse	
Junctional molecules			
CLDN-1	AAGGTGTACGACTCGCTGCT	CAGCAACAAACACACCAACC	NM_001013611.2
CLDN-2	CCTGCTCACCCTCATTGGAG	GCTGAACTCACTCTTGGGCT	NM_001277622.1
CLDN-3	GCCAAGATCACCATCGTCTC	CACCAGCGGGTTGTAGAAAT	NM_204202.1
OCDN	ACGGCAAAGCCAACATCTAC	ATCCGCCACGTTCTTCAC	NM_205128.1
ZO-1	AAGTGGGAAGAATGCCAAAA	GGTCCTTGGATCCCGTATCT	XM_015278981.2
JAM-2	AGACAGGAACAGGCAGTGCT	TCCAATCCCATTTGAGGCTA	XM_025149444.1
E-Cad	TCACGGGCAGATTTCTAT	CACGGAGTTCGGAGTTTA	NM_001039258.2
Cytokines			
IL-1b	GTACCGAGTACAACCCCTGC	AGCAACGGGACGGTAATGAA	NM_204524.1
IL-17A	CATGGGATTACAGGATCGATGA	GCGGCACTGGGCATCA	NM_204460.1
IL-22	TCAACTTCCAGCAGCCCTACAT	TGATCTGAGAGCCTGGCCATT	XM_025147965.1
TNF-a	GGCGGTGCGGCCATATAA	ATTGACGTCGTTCTGAGCGG	MF_000729.1
IFN-γ	AAGTCAAAGCCGCACATCAAAC	CTGGATTCTCAAGTCGTTCATCG	NM_205149.1
Internal control			
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217.1

The quantitative real-time polymerase chain reaction (qRT-PCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). Expression values were normalized to ribosomal protein S17 (RPS17) in the same sample and then normalized

to the control. The sequences of the primer pairs used for qRT-PCR amplification are listed in table 2-2. Samples were heated at 95°C for 5 minutes and then subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing/elongation for 1 min at 60°C. The amplifications were performed on three independent samples/groups, with triplicate reactions carried out for each sample on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.2.7. Statistical analysis

We represented our data as the mean ± standard error of the mean (SEM). Data were statistically evaluated with a one-way analysis of variance with Tukey's multiple comparison test using SPSS 20.0 software. Differences were considered significant at $p < 0.05$ (*) and $p < 0.01$ (**).

2.3. Results

2.3.1. Fecal condition and oocyst shedding

Oocyst shedding started on the 6th dpi, and the maximum numbers of fecal oocysts were $7.1 \times 10^5 \pm 4.3 \times 10^4$ oocysts/g feces on the 7th dpi (Fig. 2-1). Diarrhea was found in all *E. tenella*-infected chicks from the 3rd to the 8th dpi. Diarrhea with blood was also observed from the 4th to the 6th dpi (Table 2-3). In the control group, it was observed that the cecum had a smooth, glossy margin, cecum filled with feces, with no sign of bleeding or enteritis. The cecum in the infected group showed atrophy due to dehydration, the congested serosa vessels, and the petechial bleedings were recognized while looked grossly even without opening the cecum. Mucus and clotted blood filling the lumen of the cecum were also observed from the 4th to the 6th dpi (data not shown).

Table 2- 3. Feces observation and histomorphology lesion score evaluation.

Day post infection		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Clinical signs	**Diarrhea**	-	-	+	+	+	+	+	+	-	-
	Blooding	-	-	-	+	+	+	-	-	-	-
Lesion score	**Mean ± SEM**	ND	$0.83^{a} \pm 0.17$	$1.22^{a} \pm 0.11$	$2.44^{b} \pm 0.22$	$3.5^{c} \pm 0.10$	$3.56^{c} \pm 0.11$	$3.78^{c} \pm 0.11$	$3.67^{c} \pm 0.19$	ND	ND

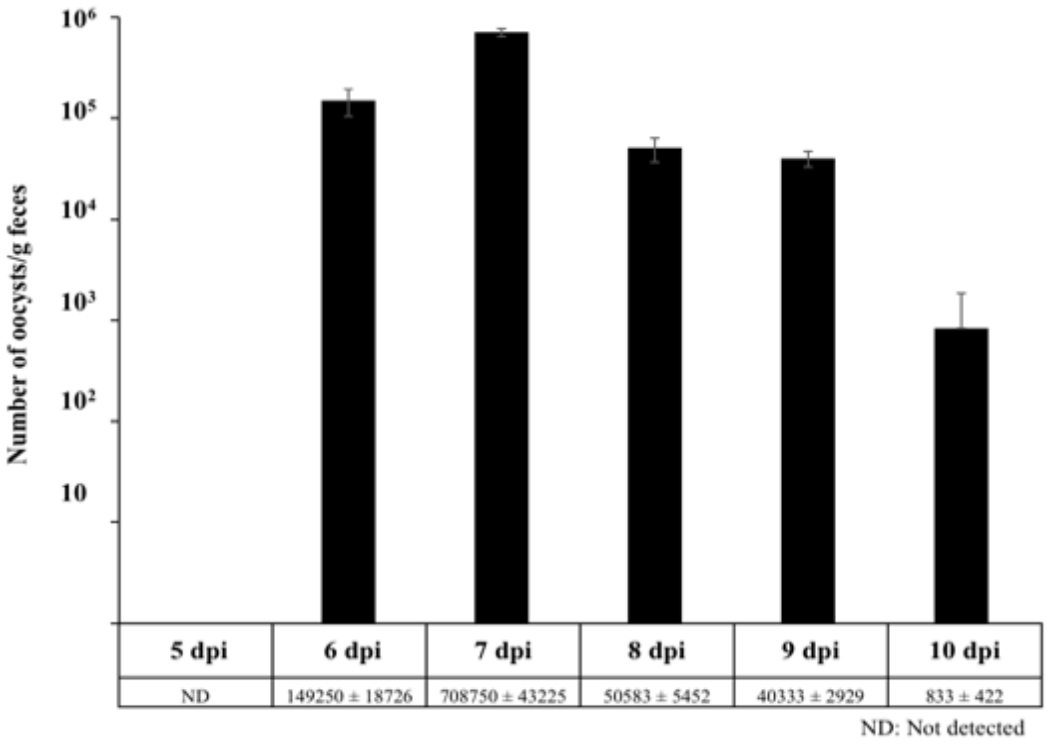


Figure 2- 1. Fecal oocyst shedding was monitored daily from 5 to 10 days post-infection (dpi). Error bars represent the standard error of the mean (SEM).

2.3.2. Histological observations

Infiltrated cells were observed through the mucosa and submucosa from the 3rd to the 6th dpi (Fig. 2-2; arrows). Infiltrated cells also extended through the mucosa and submucosa as well as marked hyperplasia of epithelial cells on the 6th dpi (Fig. 2-2D). Severe inflammation in the submucosa and the proliferation of epithelial cells of intestinal crypts were also observed on the 5th dpi and the 6th dpi (Figs. 2-2C and D). The histopathological lesion score gradually increased from the 3rd to the 5th dpi. The maximum score was 3.78 ± 0.11 on the 7th dpi (Table 2-3). We observed the epithelial monolayer to be well conserved up to the 5th dpi (Figs. 2-2A, B, and C; arrowheads). On the other hand, a small number of epithelial layers (arrowheads) and detachment of the epithelial layer on the 6th dpi (Fig. 2-2D). We observed clinical signs of villous atrophy, severe inflammation, hemorrhage, the proliferation of epithelial cells around the intestinal crypt, and epithelial desquamation in the specimens with HE-staining cecum.

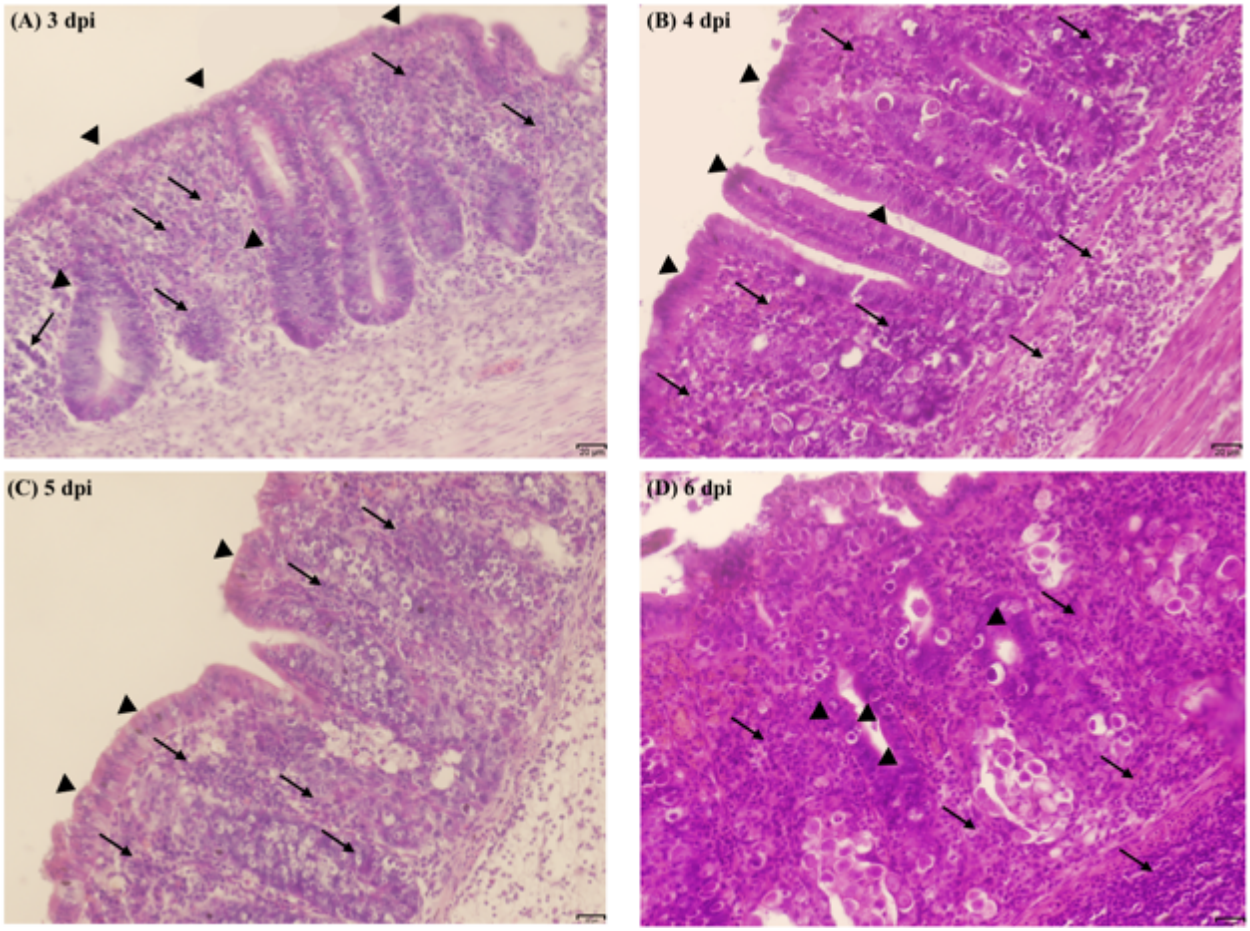


Figure 2- 2. Histopathology of HE-stained cecum sections. HE-stained specimens were observed under light microscopy. Arrows indicate the infiltrating immune cells. Arrowheads indicate the epithelial layer. (A) 3 dpi, (B) 4 dpi, (C) 5 dpi, (D) 6 dpi. Magnitude is × 200.

2.3.3. FITC-d levels in plasma

To confirm the epithelial barrier disruption caused by *E. tenella* infection, chicks were randomly divided into three groups, administrated with FITC-d, and plasma FITC-d levels were measured as described in the Materials and Methods section. The plasma FITC-d level in *E. tenella*-infected groups was significantly higher than that of the control groups during the experimental period. There was no daily increase or significant difference in the plasma FITC-d in the fasting and control groups during the experimental period (Fig. 2-3). The plasma concentration of FITC-d in the *E. tenella* group gradually increased with the course of infection, reaching the highest value on the 5th dpi, and it was significantly higher than the other days ($p < 0.05$). The plasma FITC-d levels were 0.292 ± 0.013 μg/ml for the *E. tenella*-infected group, 0.157 ± 0.001 μg/ml for the fasting group, and 0.095 ± 0.005 μg/ml for the control group on 5th dpi, especially ($p < 0.01$).

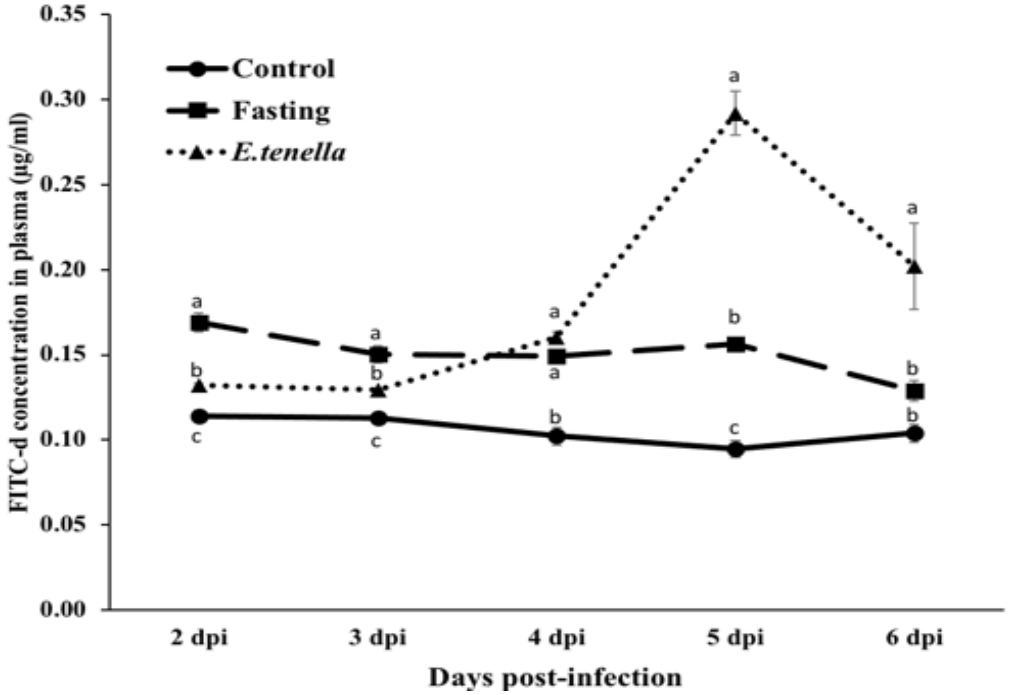


Figure 2- 3**.** The concentration of FITC-d in plasma of chicken, data are expressed as mean ± standard error of the mean. FITC-d (2.2 mg/ml) was administered by oral gavage 2.5 h before blood sample collection. [a, b, c] superscripts show the significance different at $p < 0.05$.

2.3.4. Junctional molecules mRNA expression in cecum with E. tenella

The relative mRNA expression levels of junctional molecules were determined to evaluate the relationships between the clinical signs of *E. tenella* infection and its increasing permeability. Relative mRNA expression levels of CLDN-1 showed a significant decrease at 4th dpi but were sharply raised on the 5th and 6th dpi compared with the control. CLDN-2 were significantly increased on the 3rd, 4th, and 5th dpi compared with the control group (Fig. 2-4). On the other hand, the mRNA expression levels of CLDN-3, OCDN, and ZO-1 tended to decrease throughout the experimental period compared with the control group (Figs. 2-4 & 2-5). The OCDN expression levels were significantly reduced on the 4th and 5th dpi compared with the uninfected control group (Fig. 2-5). As shown in figure 5, the relative expression level of ZO-1 mRNA was especially reduced on the 5th and 6th dpi in cecum compared with uninfected control ($p < 0.05$). The E-cadherin (E-cad) gene expression was significantly lower during the experimental period. The JAM-2 expression levels in the cecum showed no significant difference during the experimental period.

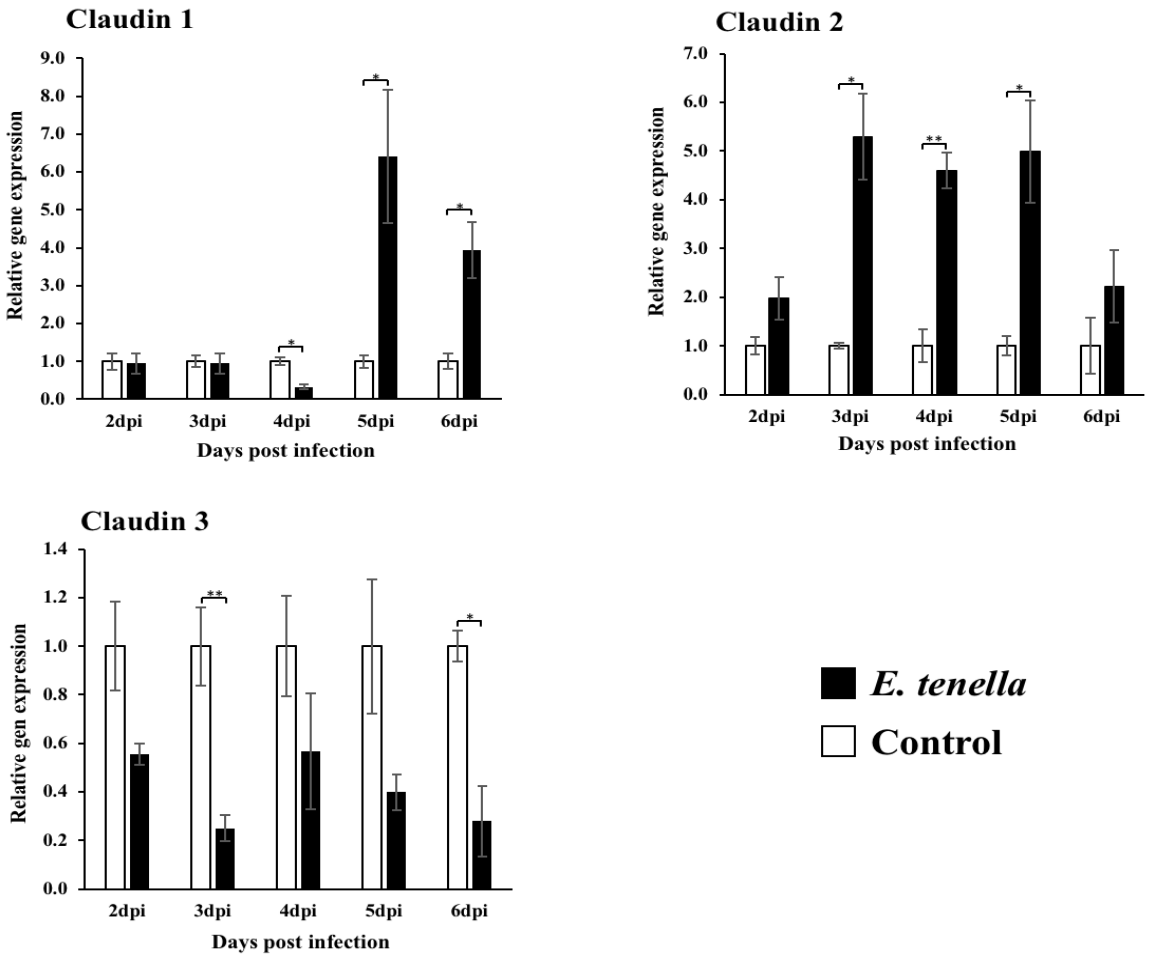


Figure 2- 4. The mRNA expression levels of claudins in ceca of chicken with *E. tenella*. Control group, chicks in this group were not treated as a control; *E. tenella*-infected group, chicks in this group were orally infected with 1×10^4 oocysts of *E. tenella*. The open column represents the control group. The filled column represents the *E. tenella*-infected group. Amplifications were performed on three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. All data are represented as the mean ± SEM and analyzed with a one-way ANOVA with Tukey's multiple comparison test using SPSS 20.0 software (* $p < 0.05$; ** $p < 0.01$).

2.3.5. Cytokines and biomarkers expression in the cecum after E. tenella infection

We also measured mRNA expression levels of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-17A, IL-22, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α (Fig. 2-6). The expression levels of IFN-γ tended to increase with the course of infection, but no statistical significance was observed. The expressions of IL-1β and IL-22 were significantly increased on the 4th to the 6th dpi in the *E. tenella*-infected chicks compared to control. The mRNA expression level

of IL-17A in infected chicks was considerably higher than normal chicks at 2nd dpi. There was no significant difference in gene expression of TNF-α.

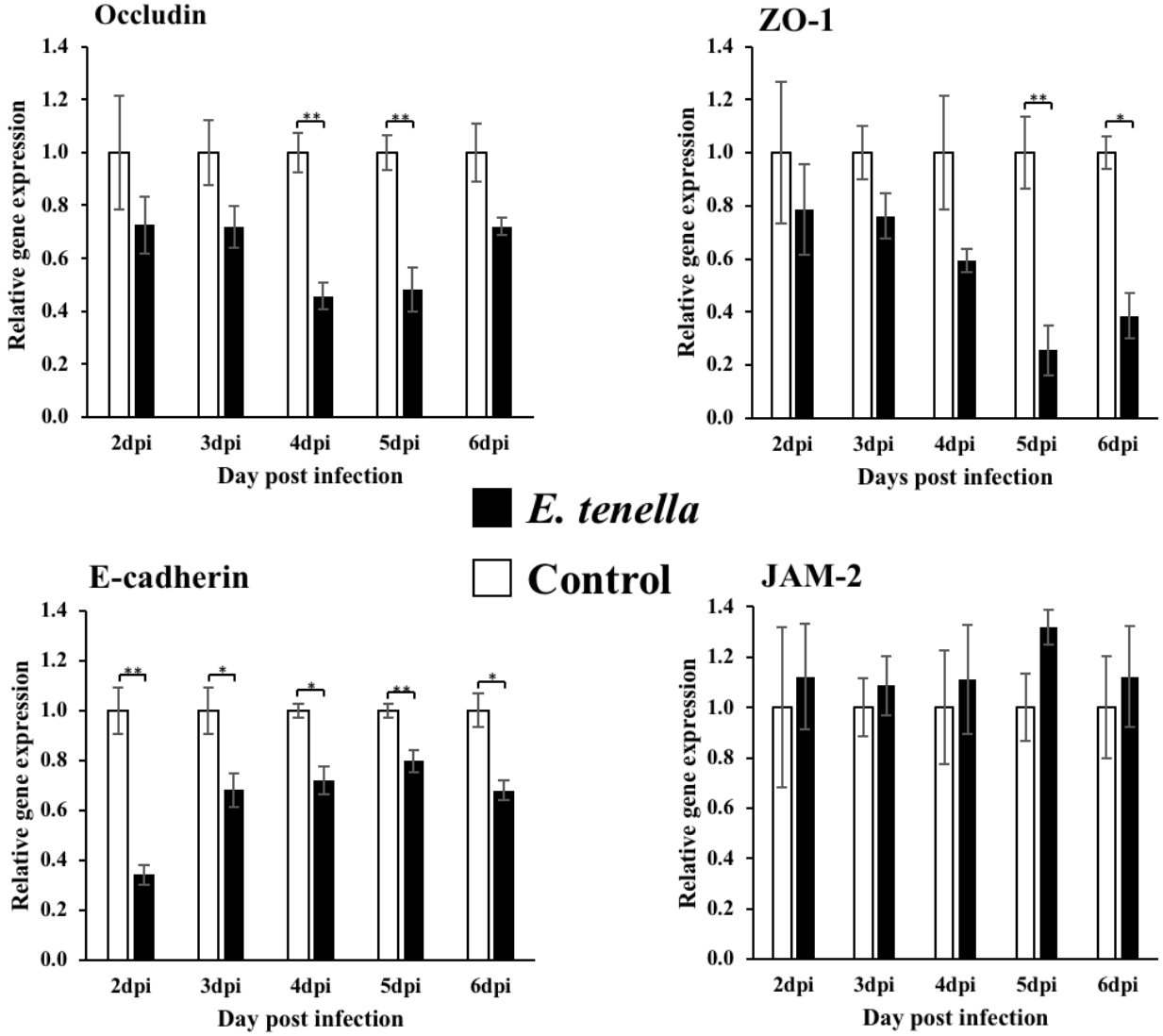


Figure 2- 5. The mRNA expression levels of Occludin, ZO-1, E-cadherin, and JAM-2 in ceca of chicken with *E. tenella*. Control group, chicks in this group were not treated as a control; *E. tenella*-infected group, chicks in this group were orally infected with 1×10^4 oocysts of *E. tenella*. The open column represents the control group. The filled column represents the *E. tenella*-infected group. Amplifications were performed on three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. All data are represented as the mean ± SEM and analyzed with a one-way ANOVA with Tukey's multiple comparison test using SPSS 20.0 software (* $p < 0.05$; ** $p < 0.01$).

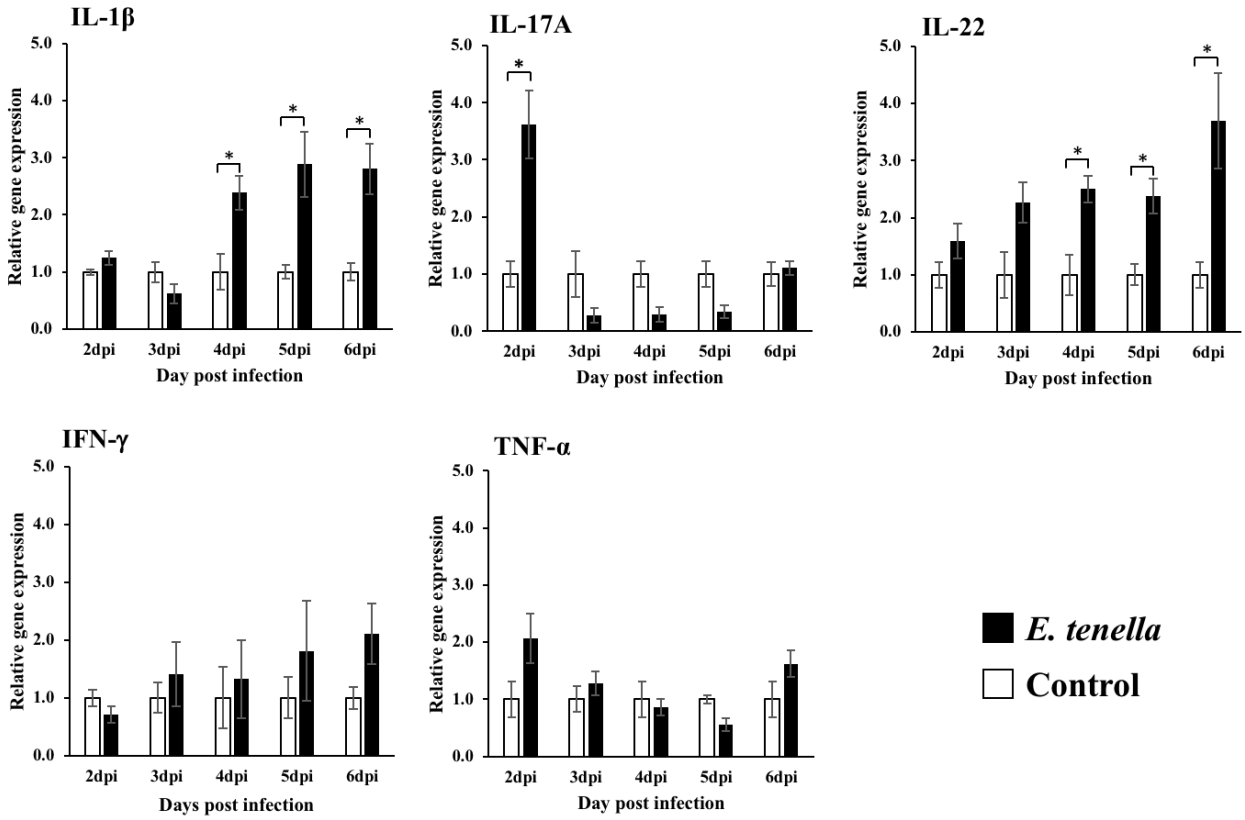


Figure 2- 6**.** The mRNA expression levels of cytokines in ceca of chicken with *E. tenella.* Control group, chicks in this group were not treated as a control; *E. tenella*-infected group, chicks in this group were orally infected with 1×10^4 oocysts of *E. tenella*. The open column represents the control group. The filled column represents the *E. tenella*-infected group. Amplifications were performed on three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. All data are represented as the mean ± SEM and analyzed with a one-way ANOVA with Tukey's multiple comparison test using SPSS 20.0 software (* $p < 0.05$).

2.4. Discussion

This study investigates the association between the expression pattern of intestinal epithelial barrier molecules and typical clinical signs in chicks with *E. tenella*. The infection of *E. tenella* is typically accompanied by severe diarrhea and bloody feces, with blood retention in the cecum (Chapman, 2014; El-Ashram et al., 2019). In this study, *E. tenella* infected chicks indicated diarrhea from 3rd to 8th dpi and bloody feces from 4th to 6th dpi as clinical signs. Similar research has reported that chicks with *E. tenella* infection had reported the emergence of bloody diarrhea from 4th to 7th dpi (Lan et al., 2016). This difference in duration of clinical signs may be due to differences in the virulence of parasite strain and the number of oocysts used because the number

of oocysts in their study was about 2.5 times compared to our study. Regarding histological observations of cecum specimens, our results show that the epithelial monolayer was well maintained at 3rd and 4th dpi. On the other hand, the lesion score, which corresponds to cecal damages, has been significantly increased from 4th dpi, reaching the maximum at 5th dpi until 8th dpi. Histopathological observations in our study indicated the loss of cecal villi, necrosis, and hemorrhage at 6th dpi on cecal mucosa, which is similar to previous reports (Sharma et al., 2015; Abdelrazek et al., 2020). Oocyst shedding started from the 6th dpi onwards, in agreement with other studies (Zhou et al., 2020; Jordan et al., 2011). Some researchers attributed clinical signs of *Eimeria* infection to damages in blood vessels due to the release of merozoite from second-generation schizonts (Burrell et al., 2019; Fernando et al., 1983; Macdonald et al., 2017). Lopez et al. (2020) have reported that the first and second generations of schizonts release merozoites at 3rd to 5th dpi and the oocyst shedding occurs at 6th dpi onwards. Especially, second-generation schizonts are large and develop deeply in the lamina propria. Therefore, once merozoites are released, blood vessels are disrupted (Burrell et al., 2019), explaining the presence of blood in feces at 4th dpi and the increases of lesion scores at 4th and 5th dpi. Findings in this study also indicates that the timing of clinical signs such as bloody feces occurred at a time that corresponds to the life cycle of *Eimeria* spp. in chickens. Hence, our results may suggest that diarrhea and bloody feces at the early stage of *E. tenella* infection are related to the destruction of intraepithelial cell junctions rather than the detachment of the epithelial layer.

To evaluate the intestinal permeability associated with the disruption of the epithelial barrier in the cecum with *E. tenella*, the levels of FITC-d in plasma of each experimental group were determined. As shown in figure 2-3, the concentrations of FITC-d in both fasting and *E. tenella*-infected groups were much higher than the control group from the 2nd to 6th dpi. Although no change was observed in FITC-d plasma levels in both fasting and the control groups between the days, plasma FITC-d levels in *E. tenella*-infected group increased with the course of infection, peaking on the 5th dpi. Teng et al. (2020) have reported gastrointestinal leakage in chickens with *Eimeria* infection was rapidly elevated at 5th dpi. Results in this study are similar to Teng et al. Further to that, results show that a significant increase of lesion score after 4th dpi was tightly associated with high plasma levels of FITC-d at 4th to 6th dpi, with rapid progression of epithelial barrier leakage during this period. These results indicate that cecal permeability increases in the *E.*

tenella infection early stage, suggesting that the epithelial barrier condition was affected as the parasite life cycle progressed.

The relations between clinal signs of *Eimeria* infection, the status of the intestinal immunity, and the epithelial barrier at the small intestine have been reported in some previous studies (Hong et al., 2006a; Park et al., 2020; Teng et al., 2020). However, molecular insights associated with clinical signs of *E. tenella* infection at the cecum have not been well evaluated in the past. Tight junction proteins are adhesive junctional molecules that link epithelial cells together and regulate epithelial barrier function, permitting the passive entry of nutrients, ions, and water, while blocking the entrance of pathogen access to underlying tissue compartments. (Groschwitz et al., 2009). Awad et al. (2017) have studied enteric pathogens and their toxin-induced disruption of the intestinal barrier by altering tight junctions in chickens. The CLDNs are the main components of tight junctions and form a seal that modulates paracellular transport in the intestinal epithelium. The CLDNs exist in two different classes, sealing claudins and pore-forming claudins (Itallie & Anderson, 2006). It is known that CLDN-1 and -3 are sealing claudins, of which increased expression leads to a tight epithelium (Haworth et al., 2005; Milatz et al., 2010). In contrast, CLDN-2 is considered as the pore-forming claudin. It creates paracellular anion/cation pores and water channels that decrease the epithelial tightness and increase the solute permeability by allowing the passage of sodium ions (France & Turner, 2017; Furuse et al., 2001). The result in figure 2-4 indicated that CLDN-1 mRNA level was first decreased at 4^{th} dpi ($p < 0.05$), but later remarkably increased at 5^{th} and 6^{th} dpi ($p < 0.05$). Teng et al. (2020) have reported that CLDN-1 gene expression increases at 6^{th} dpi in the small intestine of chicks with the *Eimeria* challenge. As CLDN-1 plays an important role in maintaining intestinal barrier integrity, the higher expression of CLDN-1 helps the chick to retrieve intestinal integrity. This could be related to acute mechanisms of repair in the injured intestinal epithelium (Slifer & Blikslager, 2020).

The expression of CLDN-2 was significantly higher at 3^{rd}, 4^{th}, and 5^{th} dpi (Fig. 2-4). Higher expressions of pore-forming proteins facilitate the paracellular permeability by allowing more luminal contents to translocate to the systemic circulation (Wada et al., 2013). Researchers reported that increased CLDN-2 is associated with barrier disruption in response to inflammation and infection (Ahmad et al., 2014; Furuse et al., 2001). This study, finds that the upregulation of CLDN-2 gene expression could induce diarrhea by draining water into the cecal lumen.

The results in this study also indicate that the CLDN-3 expression levels tended to be low during the experimental period compared with the control group, especially on the 3rd and the 6th dpi (Fig. 2-4). These results might suggest that the reduction of CLDN-3 was part of the increased paracellular permeability, resulting in the leakage of blood and other substances through this route. Several researchers also reported a decrease of sealing proteins, resulting in a leak epithelial barrier and harmful movement of luminal contents through the paracellular space (Haworth et al., 2005; Milatz et al., 2010).

The role of OCDN is to maintain the tight junction barrier and regulate paracellular pore and leak pathways (France & Turner, 2017; Itallie et al. 2010; Hossain & Hirata, 2008). Cani et al. (2009) claimed that the expression of OCDN was decreased and contrariwise correlated with the FITC-d's translocation from the intestinal tract to the bloodstream, associated with the presence of diarrhea, highlighting the importance of OCDN in maintaining the barrier function. Chen et al. (2015) has reported that the *Eimeria* parasite challenge induces gut barrier failure and inflammation in broilers by the upregulation of certain cytokines and the downregulation of OCDN in jejunum mucosa, and elevated levels of endotoxin and acid glycoprotein in their serum. In this study, the OCDN expression level tended to reduce during the experimental period and significantly decreased at 4th and 5th dpi. ZO-1 proteins help cell-cell contacts and were enriched at cell junctions in epithelial cells (Furuse et al., 2001). In this study, the expression level of ZO-1 decreased after the 3rd dpi and reduced considerably on the 5th and the 6th dpi. These findings were consistent with a previous report, suggesting that the gene expression of ZO-1 is reduced as a consequence of parasite infection (Teng et al., 2021).

According to Lechuga & Ivanov (2017), E-cad proteins are critical molecules of the adherens junction. Intestinal epithelial cells predominantly express E-cad. In this study, the expression level of E-cad was significantly reduced during the experimental period. Similarly, previous studies indicates that the infections of *E. vermiformis* (Inagaki et al., 2006) and *Cryptosporidium parvum* consequently resulted in the downward tendency of E-cad expression (Kumar et al., 2018). This data suggested that the early downregulation of E-cad gene expression could contribute to the barrier dysfunction and increased permeability in the onset of *E. tenella* infection.

The tight junction proteins can be regulated by many factors including cytokines and growth factors (Petecchia et al., 2012). In the previous research, IL-17A's neutralization increased

tissue damages in the dextran-sulfate-induced acute colitis model (Ogawa et al., 2004). This suggests that IL-17A has an essential function in maintaining the barrier function of the intestinal epithelial barrier. Lee et al. (2015) added that the absence of IL-17A increased the epithelial injury and compromised the acute colitis model's barrier function. In this study, the expression of IL-17A was suppressed at 3rd dpi onwards which coincided with the reduction of several junctional gene expressions (Figs. 2-5 and 2-6). This implies that the decreased level of IL-17A might suppress the expression of related junctional genes, including ZO-1, and therefore disrupt the barrier function in the cecum. Furthermore, IL-17A can induce the expression of pro-inflammatory cytokines such as IL-1β and IL-6 from epithelial cells and fibroblasts (Iwakura et al., 2011; Gaffen et al., 2009). In this study, the upregulations of IL-1β and IL-22 gene expressions had become significant from 4th dpi going forward, which was after the high expression of IL-17A at 2nd dpi. This indicates that the transient expression of IL-17A at the early stage of *E. tenella* infection has triggered the latter expression of IL-1β and IL-22.

Previous studies have mentioned the roles of IL-22 in protecting and regenerating cells in the gastrointestinal tract (Eyerich et al., 2017). Besides, IL-22 is also important in innate immunity and epithelial reorganization (Wolk et al., 2006). This study indicates that higher expressions of IL-22 in *E. tenella* infected chicks had been observed from the 4th to the 6th dpi. Under the inflammation process, tissues including the intestinal epithelium are exposed to multiple cytokines (Hong et al., 2006b). Among those, IL-1β is an inflammatory cytokine with diverse physiological functions and pathological significances, which plays an important role in health and disease and is often increased in the impaired intestine (Kaneko et al., 2019). It was reported that the IL-1β mRNA level was highly upregulated after parasite and bacterial infections (Laurent et al., 2001; Withanage et al., 2004). Moreover, a recent study utilizing a chicken macrophage microarray also identified that the IL-1β transcripts were elevated during experimental coccidiosis (Dalloul et al., 2007). A direct correlation was found in the mRNA expression levels between OCDN and IL-1β in this study. Therefore, it may be concluded that IL-1β impairs the intestinal tight junction barrier by decreasing OCDN expression.

IFN-γ is known to play as a mediator that enhances epithelial permeability via the disruption of tight junction complexes (Willemsen et al., 2005). Ferrier et al. (2003) has reported the relationship between stress-induced intestinal permeability and increased mucosal IFN-γ expression. Willemsen et al. (2005) has reported that the induction of CLDN-2 by IFN-γ is one of

the etiological factors of intestinal barrier dysfunction. In our experiment, the IFN-γ expression in infected chickens seems to be higher than that compared to the control from 3rd to 6th dpi, although this difference was not significant (Fig. 2-6). Previous research has also reported that TNF-α plays an important role in the proinflammatory cytokines-induced intestinal barrier disruption (Ma et al., 2004; Graham et al., 2006; Ye et al., 2008). However, in this study, TNF-α was also no significant difference between control and infected chickens. It is not known whether this derives from the diverse attack mechanisms of the parasite itself or the result of the host mechanism to rebalance the barrier function experiments are needed to further explore this matter.

2.5. Conclusion

This is the first study to describe daily changes in intestinal junctional gene expressions upon *E. tenella* infection in chick cecum. The findings in this study, suggest that the expression of junctional molecule genes are related to clinical signs such as diarrhea and bloody feces in chicks infected with *E. tenella*. The disruption of barrier function via downregulation of CLDN-1, CLDN-3, E-cad, OCDN, and ZO-1, but increased CLDN-2, could contribute to *E. tenella* infection-induced diarrhea. Furthermore, this study, reports a link between the high levels of pro-inflammatory cytokines and junctional molecules related to the epithelial barrier and intestinal permeability. Insights on the inflammation-dependent alterations of junctional gene expressions will provide new ideas in the development of therapeutics for improving mucosal healing and barrier function in *E. tenella* infection. Further *in vitro* studies will be needed to verify the relationship between *E. tenella*-induced alteration of apical junctional complexes proteins and responses of host epithelial cells and their impacts on barrier function.

CHAPTER III

THE EFFECTS OF *LACTOBACILLUS ACIDOPHILUS* L-55 ADMINISTRATION TO CHICKEN INTESTINE UNDER THE *EIMERIA TENELLA* INFECTION MODEL

3.1. Introduction

Avian coccidiosis is a significant endemic disease in poultry that has a considerable economic impact on producer profitability. The disease is caused by ingestion of the infective oocysts of various species of the protozoan parasite of the genus *Eimeria*. The annual financial loss to the poultry industry as a result of coccidiosis worldwide has been estimated at around $3 billion due mainly to prophylactic or therapeutic in-feed medications and also as a result of the disease impact on poultry health (Michels et al., 2011; Dalloul & Lillehoj, 2006).

Eimeria species multiply in the chicks intestinal tract, causing considerable tissue damage and, subsequently, impaired nutrient absorption and blood loss. In addition, it can cause diarrhea, dehydration, mortality, a transient drop in egg production in laying flocks and increased susceptibility to other diseases such as necrotic enteritis (Lillehoj et al., 2004). The severity of the infection depends on the number of ingested oocysts and the immune status of the birds; however, the disease is self-limiting. Chickens are the natural host of seven species of eimeria, with *Eimeria tenella*, *Eimeria maxima* and *Eimeria acervulina* being the most common species. All ages and breeds are susceptible to infection, but outbreaks of coccidiosis usually take place between two and six weeks of age.

Currently, drugs and live vaccines are the two main control measures for the disease. Nevertheless, these drugs, on some occasions, can be toxic to the birds and also have to be rotated from time to time to minimize the possibility for eimeria species to develop resistance to them. More recently, the poultry industry has been under pressure to reduce reliance on antimicrobials, including anticoccidial drugs, despite the global acceptance and success of these drugs. The pressure comes primarily from the high costs of these antimicrobials, which contribute to the cost of disease control, besides public health concerns and demands for drug residue-free products. Moreover, the development of resistance or decreased sensitivity of eimeria species to chemotherapeutic agents has been reported for several years now from different parts of the world, and this resistance has caused significant reductions in drug effectiveness. Although some coccidia develops less resistance to some drugs, long-term exposure eventually leads to a loss of sensitivity and development of resistance to the drug.

Coccidia vaccines have achieved relative successes in controlling the disease, especially in broiler breeders; however, they have not yet achieved satisfactory levels, particularly in broilers as their use is limited by the possibility of adverse effects on feed efficiency and high production costs,

especially when these vaccines include more than one *Eimeria* species. Yet another factor limiting the use of vaccines against coccidia is the inclusion of several species of Eimeria in one vaccine, which can cause further depression in weight gain and feed conversion and a potential vaccine failure. Taken together, due to increasing problems with prolonged drug usage and vaccines, alternative strategies are needed for more effective and safer control of coccidiosis in chickens (Dalloul et al., 2006; Williams, 2006).

Probiotics are live microbial supplements that have beneficial effects on the host animal by improving the intestinal microbial balance (Fuller, 1989). Variety of microbial species, including *Lactobacillus*, *Streptococcus*, *Aspergillus*, *Candida*, *Saccharomyces* and *Bacillus* have beneficial effects on performance, microflora, inhibiting pathogens and modifying the immune system in poultry (Zulkifli et al., 2000; Kalavathy et al., 2003; Kabir et al., 2005; Karimi Kivi et al., 2015). By using probiotics, stimulation the acquired immune, changes in the microbiome of the cecum and the production of inhibiting metabolites such as organic acids are the possible mechanisms to reduce pathogens in the digestive tract (Neal-McKinney et al., 2012). As probiotics, Lactobacillus species have been used to improve the intestinal health and growth performance of poultry. Studies have shown that *Lactobacillus* species isolated from different parts of the gastrointestinal tract of poultry are able to inhibit invasion of *Eimeria tenella*, *in vitro* (Tierney et al., 2004) as well as *in vivo* studies confirm that probiotics reduce the intestinal lesions caused by *Eimeria* (Lan et al., 2004; Lee et al., 2007*).* In a recent study, a probiotic compound containing 4 strains of lactic acid (*L. acidophilus*, *L. fermentum*, *L. Planetarium* and *Enterococcus faecium*) significantly reduced the intestinal ulcers caused by *Eimeria*, decreased the expression level pro-inflammatory cytokines such as interleukin (IL) -1β, IL-6, and interferon (IFN) -γ significantly increased the gene expression of the anti-inflammatory cytokine IL-10 (Chih-Yuan et al., 2016).

Despite the fact that several studies have shown parasite disease prevention or immune enhancement resulting from oral administration of probiotics, few studies are available on their specific effects on the gut defense mechanisms in chickens, particularly in coccidiosis. The present work was conducted to help characterize some of these actions; the specific objectives were to determine the effects of a *Lactobacillus acidophilus* LaL-55 to the clinical signs and intestinal condition associated the expression of junctional genes barrier-related in chickens challenged with *E. tenella*, and the potential protection it might provide to the chicks against a coccidial challenge.

3.2. Material and methods

3.2.1. Parasite

The *E. tenella* NIAH strain, which is virulent and maintained at the Laboratory of Animal Physiology in Okayama University (Okayama, Japan), was used. *E. tenella* oocysts were purified by the sugar flotation method, sporulated at 28 °C in 2.5% potassium dichromate, and stored at 4 °C before use.

3.2.2. Animals

Eggs (White Leghorn) were purchased from Kui Poultry Co., Ltd. (Mihara, Hiroshima, Japan). Eggs were incubated at 37.7°C ± 1°C until hatching. After hatching, chicks were maintained at the coccidian-free room and fed and water *ad libitum*. The animals were housed at a constant temperature (27°C ± 1°C) with a 12 h dark/light cycle. All procedures were approved by the Animal Care and Use Committee of Okayama University (OKU-2020201) and conducted in compliance with the Policy on the Care and Use of the Laboratory Animals of Okayama University. Animal care and experiments were also carried out in accordance with the guidelines for animal experiments at Okayama University. The body weights of chicks were measured daily during this experiment.

3.2.3. LaL-55 administration and E. tenella oocysts inoculation

Chicks (n = 18) were randomly divided into 4 groups: including 2 control groups (with or without LaL-55) and 2 *E. tenella* infected groups (with or without LaL-55). For the chicks in LaL-55 group, the dose of freeze-dried LaL-55 powder 0.75 mg/100 g body weight was suspended in 1 mL distilled water and compulsively administered orally to chicks daily from 7 days of age to the end of experiment (29 days old). Freeze-dried powder of LaL-55 was prepared as reported previously (Sunada et al., 2007). The chicks in the *E. tenella* group were orally inoculated with mature sporulated oocysts of *E. tenella* (1 x 10^4 oocysts/chick) at 14 days old. At 5 dpi, three chicks were randomly picked up, anesthetized using Pentobarbital sodium salt (Tokyo Chemical Industry. Co., Ltd., Tokyo, Japan), and sacrificed by cervical dislocation for cecum collection. One of the ceca was immediately frozen at -80 ˚C for gene expression analysis and another was separated 3 pieces (proximal, medial, and distal regions). Each of the tissue pieces was fixed with 10% formaldehyde for histopathological observation.

3.2.4. Fecal collection and oocysts counting

Feces were daily collected from 4 to 15 dpi for oocysts counting. Oocysts per gram of feces were counted by the fecal flotation method using the saturated sucrose solution (Ho et al., 2021). Briefly, fecal samples (2 g/tube) were mixed thoroughly with 10 mL of distilled water, followed by centrifuging at 2,500 rpm for 5 min at room temperature. The supernatant was then discarded, and 10 mL of the saturated sucrose solution was added to the tubes, mixed thoroughly, and centrifuged at 2,500 rpm for 5 min at room temperature. The supernatant was transferred to other 15 mL centrifuge tubes and mixed well. The supernatant (10 μL) was dropped on the slide glass, covered using cover glass. Finally, the oocysts were counted using light microscopy (triplicate/tube).

3.2.5. Histopathological observation

The middle part of formaldehyde-fixed ceca was removed and embedded in paraffin, sectioned at 6 μm thickness, and de-paraffinized. Sectioned specimens were stained using a hematoxylin-eosin (HE) solution. HE specimens (6 specimens/chick, 200 μm interval) were observed under the light microscope (Olympus FSX100, Olympus, Tokyo, Japan) to evaluate the histological score and parasite burden level (magnification, x 200). The inflammation levels of ceca were evaluated using a 0 to +4 scoring system as described previously (Table 3-1) (Erben et al., 2014).

Table 3- 1. The scoring system for evaluating inflammation level.

Inflammatory cell infiltration		**Epithelial changes**	**Mucosal architecture**	**Score**
Severity	**Extent**			
Minimal	Mucosa	Minimal Hyperplasia		1
Mild	Mucosa and submucosa	Mild Hyperplasia		2
Moderate	Mucosa, submucosa, sometimes transmural	Moderate Hyperplasia		3
Marked	Mucosa, submucosa, often transmural	Marked Hyperplasia	Ulceration, Crypt loss	4

3.2.6. Gene expression analysis

The mRNA levels for junctional genes (CLDN-2, CLDN-3, OCDN, ZO-1, ECAD) were determined using qRT-PCR. The sequences for the primer pairs used in this experiment are listed

in table 3-2. The RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was treated with DNase I (Takara Bio, Otsu, Shiga, Japan), and the cDNA synthesis was carried out using oligo (dT_{18}). Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). The RPS17 amplification was used as an internal control. The samples were heated at 95°C for 1 min and then subjected to 40 cycles of denaturation at 95°C for 15 s and annealing/elongation for 1 min at 60°C. Amplifications were performed on three independent samples per group, with triplicate reactions for each sample on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Table 3- 2. The sequence of primer pairs used for amplification of target genes.

Gene Name	Primer sequence (5' to 3')		Accession No.
	Forward	Reverse	
Junctional molecules			
CLDN-2	CCTGCTCACCCTCATTGGAG	GCTGAACTCACTCTTGGGCT	NM_001277622.1
CLDN-3	GCCAAGATCACCATCGTCTC	CACCAGCGGGTTGTAGAAAT	NM_204202.1
OCDN	ACGGCAAAGCCAACATCTAC	ATCCGCCACGTTCTTCAC	NM_205128.1
ZO-1	AAGTGGGAAGAATGCCAAAA	GGTCCTTGGATCCCGTATCT	XM_015278981.2
E-cad	TCACGGGCAGATTTCTAT	CACGGAGTTCGGAGTTTA	NM_001039258.2
Internal control			
RPS17	AAGCTGCAGGAGGAGGAGAGG	GGTTGGACAGGCTGCCGAAGT	NM_204217.1

3.2.7. Statistical analysis

Data were represented as the mean ± standard error of mean (SEM). Data were statistically evaluated by one-way analysis of variance (ANOVA) with Turkey's test using SPSS 20.0 software.

3.3. Result

3.3.1. Fecal oocyst shedding

Fecal oocyst shedding was monitored daily from 4 to 15 dpi (Fig. 3-1). Oocyst shedding started at 6 dpi in both the infected groups. At 7 dpi, the maximum numbers of fecal oocysts were

recorded as $1.26 \times 10^6 \pm 4.4 \times 10^4$ oocysts/g feces for the infected without LaL-55 group and $1.22 \times 10^6 \pm 4.5 \times 10^4$ oocysts/g feces for the infected with LaL-55 group. The number of fecal oocysts in the infected with LaL-55 group was found to be significantly lower than that in the infected control group from 9 to 10 dpi ($p < 0.01$). There were no detected oocysts shedding in both infected group from 11 dpi to 15 dpi.

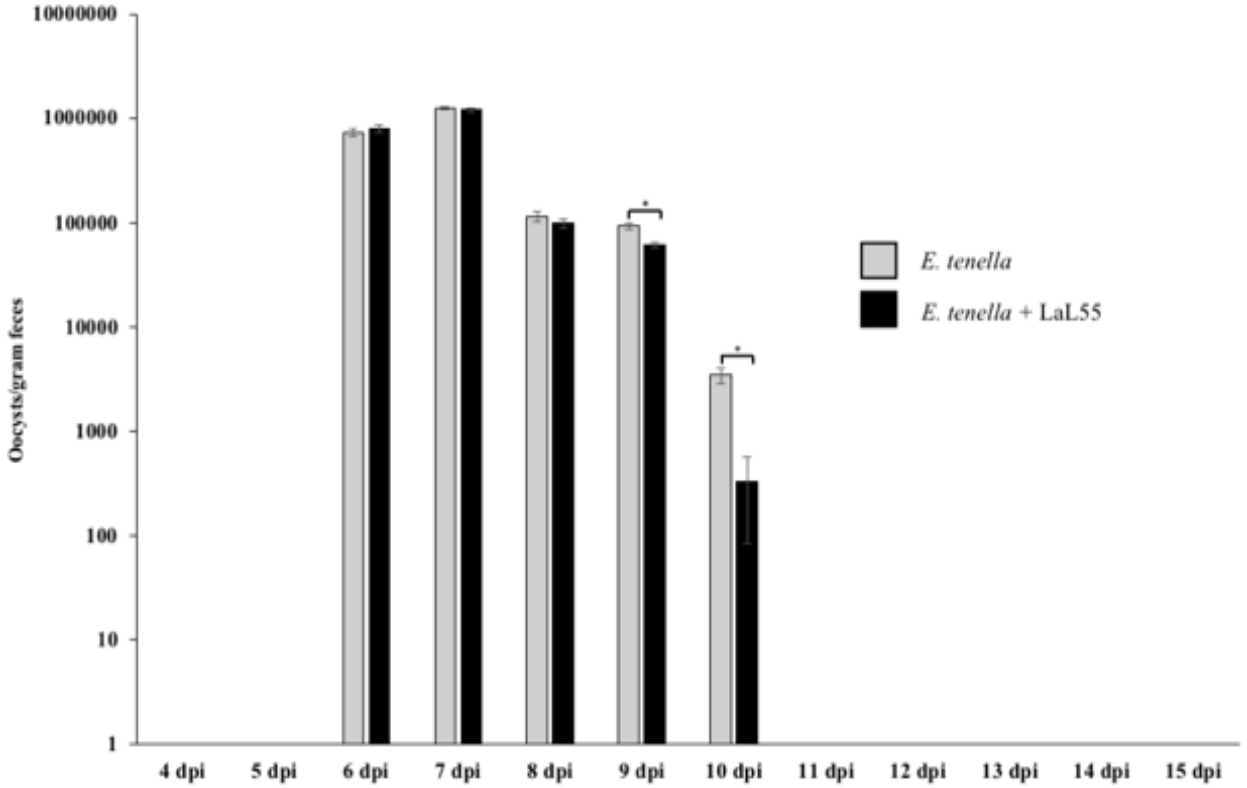


Figure 3- 1. Fecal oocyst shedding was monitored daily from 4 to 15 days post-infection (dpi). All data are represented as the mean ± SEM and analyzed with a one-way ANOVA with Tukey's multiple comparison test using SPSS 20.0 software (* $p < 0.01$).

Table 3- 3. Fecal oocyst shedding (occysts/gram feces)

	5 dpi	**6 dpi**	**7 dpi**	**8 dpi**	**9 dpi**	**10 dpi**	**11 dpi**
E. tenella	N/D	747917 ± 64188	1268500 ± 44459	117083 ± 13401	94167 ± 6133	3500 ± 619	N/D
E. tenella **+ L55**	N/D	807500 ± 67602	1222917 ± 45854	99833 ± 9849	62333 ± 3338	333 ± 247	N/D
p		0.53	0.49	0.32	**0.001**	**0.001**	

3.3.2. Clinical sign

All the infected chicks showed clinical signs of coccidiosis such as huddling together, ruffled feathers, and depression. Mortality was absent in all groups. Diarrhea was observed from the third to seventh day after *E. tenella* challenge in infected group without LaL-55. In the infected chicks with LaL-55, diarrhea also appeared in 3 dpi, but it was finished at 5 dpi. The feces containing blood was observed from 4 dpi in both group of infected chicks, but in the chicks infected with LaL-55, the bloody feces were non observed at 6 dpi, but in the chicks infected without LaL-55, the blood feces were still recorded at 6 dpi, it just does not appear from 7 dpi (Table 3-4). Non-observed of clinical signs such as diarrhea or feces containing blood in chicks of both uninfected groups through-out the whole experiment.

Table 3- 4. Clinical signs of chicken with *E. tenella* infected

Days post infection		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Diarrhea	***E. tenella***	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
	***E. tenella* + LaL55**	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Bloody Feces	***E. tenella***	-	-	-	++	+++	+	-	-	-	-	-	-	-	-	-
	***E. tenella* + LaL55**	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-

3.3.3. Histological evaluation

The histological score in the cecum was similar in both infected groups (Table 3-5). Similarly, the parasite burden scores in the infected chick with and without LaL-55 groups were not significantly different, it showed that 3.67 ± 0.17 and 3.89 ± 0.11 at 5 dpi, respectively. Severe inflammation in the submucosa and the proliferation of epithelial cells of intestinal crypts were observed in both groups with *E. tenella* infection (Fig. 3-2). The presence of high numbers of schizonts (asterisk) and infiltrated cells was also observed through the mucosa and submucosa in both infected groups with/without LaL-55 (arrows). However, the epithelial monolayer is still well conserved (arrowheads) even in the infected group. There are not any abnormalities in histopathology observation in both uninfected groups.

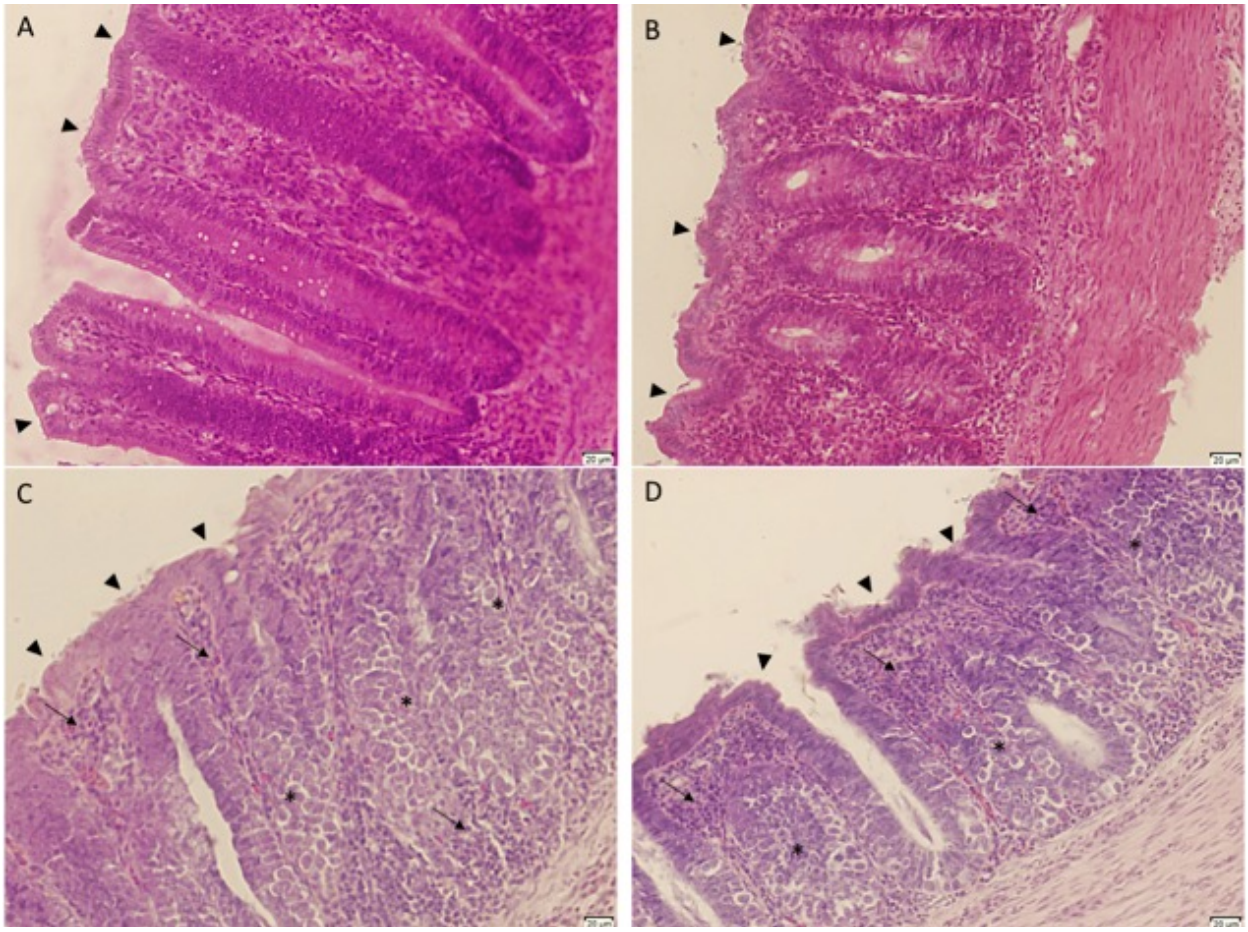


Figure 3- 2. Histopathology of HE-stained cecum sections at 5 dpi. Arrows indicate the infiltrating immune cells. Arrowheads indicate the epithelial layer. Asterisk indicate the schizonts. (A) Control; (B) Control with LaL-55; (C) Infected; (D) Infected with LaL-55. Magnitude is × 200.

Table 3- 5. Histopathology lesion and burden score.

5 dpi	**Histopathology scores**		**Burden scores**	
	E. tenella	L55 + *E. tenella*	*E. tenella*	L55 + *E. tenella*
Cecum	3.78 ± 0.15	3.56 ± 0.18	3.67 ± 0.17	3.89 ± 0.11
Small intestinal	0.67 ± 0.17	0.56 ± 0.18	-	-

In the small intestinal, the histological just showed low lesion scores in 2 infected groups, 0.67 ± 0.17 and 0.56 ± 0.18 respectively. No burden score in the small intestinal. The uninfected groups were not evaluated the lesion score in cecum.

3.3.4. Gene expression analysis

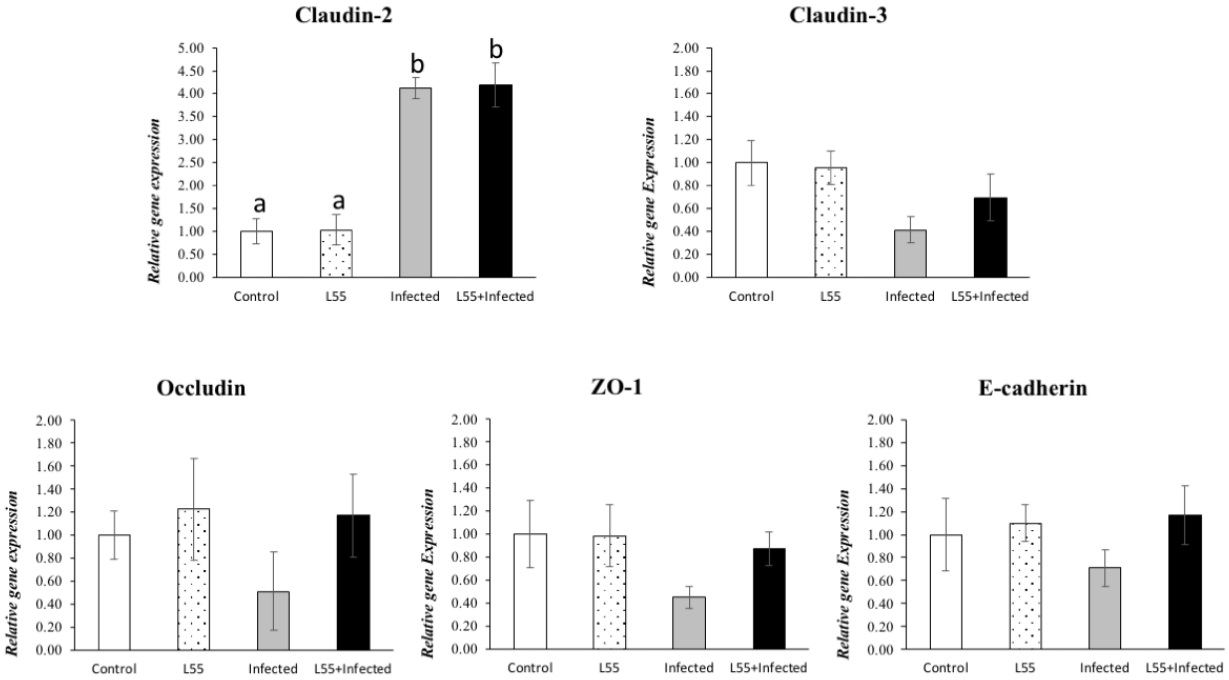


Figure 3- 3. The mRNA expression levels of junctional gene in ceca of chicken with *E tenella* infection. Control group (n=3), chicks in this group were not treated as a control; L55 group (n=3), chicks in this group were oral administration LaL-55; Infected group (n=6), chicks in this group were orally infected with 1×10^4 oocysts of E. tenella; L55+Infected group (n=6), chicks in this group were orally infected with 1×10^4 oocysts of *E. tenella* and oral administration LaL-55. Amplifications were performed on three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. All data are represented as the mean ± SEM and analyzed with a one-way ANOVA with Tukey's multiple comparison test using SPSS 20.0 software (^{a,b} Different superscripts show the significance between groups at $p < 0.01$).

The qRT-PCR analysis was used to examine the expression of junctional genes in the ceca among the chick groups (Fig. 3-3). The relative mRNA levels of CLDN-2 were increased ($p < 0.01$) in both infected groups as compared to both non-infected groups. However, there was no difference in the expression pattern of CLDN-2 between the group of infected chickens administrated LaL-55 and the infected control group. Despite the fact that the mRNA levels of CLDN-3, OCDN, ZO-1, and E-cad among all groups showed no statistical difference, there was a tendency that the expression levels of these genes in both infected groups were lower than the two uninfected groups. Additionally, when comparing two infected groups, the transcript levels of these genes in chicks administrated with LaL-55 were slightly higher than that in chicks without supplemented probiotic, though the differences were not statistically significant ($p > 0.05$).

3.4. Discussion

Adverse effects of coccidiosis include bloody diarrhea, intestinal lesions, depressed growth rate and, sometimes, high mortality. In this study, we used an established coccidiosis model of chicken to evaluate the effect of continuous LaL-55 administration on the development of *E. tenella* parasite. Daily changes in clinical signs and histopathology lesion score within the cecum were presented in table 3-3 and 3-4. Fecal oocyst shedding following *E. tenella* infection was used as an assessment of susceptibility to the pathogen of LaL-55 (Fig. 3-1). The gene expression result of intercellular junctions such as CLDN-2, CLDN-3, OCDN, ZO-1, and E-cad in 5 dpi are shown in figure 3-2.

The observation of clinical signs shows the similarity results to our series of experiments, the typical signs of coccidial infection were started in appearance of diarrhea in the infected chicks at 3 dpi and stop at 7 dpi, from 4 to 6 dpi, all the infected chickens showed bloody diarrhea. Mild signs were observed in the group supplemented with the LaL-55 probiotic before infection, with all clinical signs was stopped at 5 dpi in this group. Although the clinical signs in coccidiosis showed the differently in levels between two infected groups, no statistically significant difference was found in the incidence of lesions score. Dissimilar to present results, Lee et al. (2010) reported that birds given a strain of a *Bacillus*-based direct-fed microbial had significantly lower lesions scores in the gastrointestinal tract than birds given the non-supplemented diet following an *E. maxima* challenge. Studies investigating necrotic enteritis in broilers found birds given 2 different blends of direct-fed microbials had significantly reduced intestinal lesions due to necrotic enteritis than birds in the positive control (McReynolds et al., 2009). Fewer and less severe lesion scores are indicative of less damage to the epithelium of the intestine, leading to infected birds having a greater chance of recovery from disease. Numerous studies have found probiotic supplementation leads to significant reductions in numbers of other intracellular pathogens present in the intestine, such as *Salmonella enteritidis* and *Campylobacter jejuni* (Higgins et al., 2007; Ghareeb et al., 2012).

In the present study, the cecum sample was collected and analyzed at 5 dpi, this is the time point for the most severe effect in the intestine mucosa in *E. tenella* infection time course (Huang et al., 2018; Yan et al., 2015). The presence of high numbers of schizonts and severe tissue damage in the caeca indicated the severity of infection due to *E. tenella*. Histopathological examination of the affected ceca showed similar findings with those reported by Zhou et al. (2010), who described the most pathogenic stage caused by E. tenella as the second generation schizont, which caused

excessive tissue damage, bleeding, disruption of the caecal glands, and destruction of the mucosa and muscularis layer. However, the dose of LaL-55 used in this experiment may not have been sufficient to mitigate extensive damage to the site of infection, resulting in the not difference in lesions in the ceca between two groups with and without probiotic. The pathogen load reductions could be due to multiple mechanisms of action employed by direct-fed microbials, depending on strains present in various products employed in those studies. Ultimately, the reduction in the presence of intracellular pathogens is indicative of a healthier intestine, with minimal damage done to the epithelium. An intact intestinal epithelium serves as the vital barrier preventing entry of potential pathogens and results in proper nutrient absorption and utilization, leading to optimal health and performance of the chick. Lesion scores can provide information on *Eimeria* species responsible for infection, as well as the degree of intestinal damage at a particular moment, but the limited window in which to observe peak incidence of lesions is relatively narrow and dependent upon the incubation period of the species (Chapman et al., 2013). Lesion scores have also been shown to be affected by isolate, resulting in score variability and infection location based on *Eimeria* strains present (Barrios et al., 2017, El-Sherry et al., 2019), limiting the information they provide about infection severity and efficacy of treatment. Therefore, the incorporation of lesion score should be used in conjunction with other measures of coccidial infection, such as oocysts per gram feces, to provide an accurate representation of the effects of certain prevention or treatment methods.

Fecal shedding of oocysts represents another parameter for measuring the disease impact of *Eimeria*. The reduction of oocysts is also used as a tool to determine the protective indices of different anti-parasite strategies against *Eimeria* invasion. In this study, oocysts shed in feces collected on days 4 to 15 post infection were enumerated (three birds per group). The number of fecal *E. tenella* oocysts was shown similarly in both infected groups at 6 dpi, 7 dpi and 8 dpi, the significant difference in the oocysts shedding between these two groups just recorded from 9 dpi, with the average 94×10^3 oocysts/gram feces in the infected control group but was reduced ($p < 0.01$) to 62×10^3 oocysts/gram feces in the probiotic-treated group. Noticeable, at 10 dpi, the reduction of oocysts shedding was more than tenfold when compare between these groups, 3500 oocysts/gram feces in infected control group and 333 oocysts/gram feces in infected group used LaL-55, a clear indication of improved resistance to *E. tenella* infection, though this phenomenon occurred at the later of *E. tenella* life cycle. Not surprisingly, the infected chicks receiving a

probiotic dose of LaL-55 shed fewer oocysts in the feces than the infected control chicks. Corroborating our findings, Dalloul et al. (2003, 2005) found that broilers provided a *Lactobacillus*-based probiotic in the feed shed significantly fewer *E. acervulina* oocysts compared with the challenged control. A reduction in oocysts shed in the feces indicates improved resistance of the bird to *Eimeria* species infection. The exact protection mechanism is not clear; the doses of parasite challenge and the doses of probiotic are the possible factors to think of. It could be partially attributed to the late stimulation of the immune elements within the intestine by probiotic bacteria, leading to a slower immune response to *Eimeria*.

Although the precise mechanisms underlying the beneficial effects of probiotics are unclear, one of the proposed modes of action of probiotics is their pathogen interference and antagonistic activity, whereby probiotic strains inhibit the growth and colonization of other microorganisms, such as pathogens (Shokryazdan et al., 2014). This could be due to competitive exclusion by competing for nutrients and attachment sites on the intestinal epithelial wall, or production of antimicrobial substances by probiotic strains or synergy of both actions (Kizerwetter et al., 2005; Willis et al., 2007). As a result, probiotic strains can help to maintain the gut health by providing a beneficial microbial balance in the gastrointestinal tract, and a healthy, well functioning gut with reduced digestive disorders would ensure better utilization and conversion of feeds, resulting in improved growth and vitality of the animal (Fuller et al., 2001). Most of previous studies results converge towards the existence of a protective, though partial effect, by these different probiotic preparations against avian coccidiosis, the mechanisms involved remain currently elusive, with variable data concerning the reduction in oocyst shedding, the lesion score and the levels of intestinal epithelium barrier integrity.

The disruption of the intestinal epithelial barrier may lead to diarrhea and bloody feces that are frequently occurred in chickens with inflammatory intestinal diseases, particularly in coccidiosis (Groschwitz & Hogan, 2009). Understanding factors that modulate gut barrier integrity may yield insights into strategies to prevent these diseases. It is known that the tight and adherens junction proteins play important roles in regulating epithelial paracellular permeability (Betanzos et al., 2003). Our previous report demonstrates that the chicken intestinal barrier function was altered during the first week of *E. tenella* post-infection, which was caused by the modulation of junctional molecules (Pham & Hatabu, 2021). Here we report the investigation to know whether

LaL-55 bacteria help to protect the intestinal barrier by adjusting gene expressions of junctional molecules.

CLDN-2 is considered as the pore-forming claudin which creates paracellular anion/cation pores and water channels that decrease the epithelial tightness and increase the solute permeability by allowing the passage of sodium ions (France & Turner, 2017; Furuse et al., 2001). The higher expression of pore-forming proteins facilitates paracellular permeability by allowing more luminal contents to translocate to the systemic circulation (Wada et al., 2013). In this study, The relative mRNA levels of CLDN2 were increased ($p < 0.01$) in both *E. tenella* infection groups compared to both uninfected groups. In both infected groups, there was no difference in the expression pattern of CLDN2 between the chickens with and without LaL-55 administration. Researchers reported that an increased level of CLDN-2 is associated with barrier disruption in response to inflammation and infection (Ahmad et al., 2014; Furuse et al., 2001). Ewaschuk et al., (2008) reported that *Bifidobacteria infantis* are effective in reducing gut permeability and inflammation in T84 human epithelial cells by downregulating CLDN-2 expression. The expression of the CLDN-2 gene was not changed in this study after probiotic supplement can be due to the difference in used doses of LaL-55. Further study needs to be conducted to investigate the dose dependence of probiotic towards the expression of the CLDN-2 gene.

The mRNA levels of CLDN-3, OCDN, ZO-1, and E-cad among all groups showed no statistical difference, but there was a tendency that the expression levels of these genes in both infected groups were lower than the two uninfected groups. Many studies reported that the transcript levels of these genes were decreased in the gut during inflammatory infection as compared to the control (Du et al., 2016, Teng et al., 2000). In this study, the mRNA levels of CLDN-3, OCDN, ZO-1 and E-cad in infected chicks administrated with LaL-55 were slightly higher than those in infected chicks without LaL-55 ($p > 0.05$). Although there is no statistical difference among data, these results may suggest that the administration of LaL-55 to *E. tenella* infected chicks may positively influence gut barrier function by increasing the expression of related genes, which needs to be examined further for confirmation. Similarly, Park et al. (2020) showed that the upregulation of these genes improved gut barrier function in the chicken intestine after *E. acervulina* infection. The administration of probiotic bacteria subsequently increases CLDN-3 gene expression, reduces inflammation, and reverses tight junction disruption in the intestinal epithelium (Patel et al., 2012). Ewaschuk et al. (2008) found that orally administered conditioned

medium from *Bifidobacteria infantis* was effective in reducing the colonic permeability and attenuating inflammation in a mouse model of colitis by enhanced protein expression of OCDN. A previous study shows that *Lactobacillus* could prevent the adhesion of pathogens to host cells and protect the mucosal barrier by increased expression of E-cad (Yu et al., 2012). On the other hand, Wang et al. (2018) report that there was no significant difference in OCDN and E-cad at the mRNA level when investigating the effect of *L. plantarum* on the gut permeability in the presence or absence of *Salmonella* infection in chicken. The discrepancies among reports might be due to the use of different probiotics against different pathogen models. Regarding ZO-1, Li et al. (2018) reported that the relative mRNA level of ZO-1 in the intestine was not significantly affected by *C. perfringens* infection or *L. acidophilus* treatment.

Overall, our results suggested that LaL-55 administration might partially improve the resistance against *E. tenella* in chicks by mitigating clinical signs and reducing the number of oocysts shedding. Although in this study, LaL-55 administration did not confer a statistically significant recovery upon intestinal barrier disruption by *E. tenella* infection, the moderate upregulation of CLDN-3, OLDN and E-cad gene expression in infected chicks with LaL-55 supplement may help to maintain the intestinal barrier integrity. Further study needs to be carried on to fully understand the beneficial effects of *Lactobacillus*-based probiotics in this aspect.

CHAPTER IV

THE EFFECTS OF *LACTOBACILLUS ACIDOPHILUS* L-55 ON THE GENE EXPRESSION OF CYTOKINES AND MASTER IMMUNE SWITCHES IN THE ILEUM AND SPLEEN AFTER NEWCASTLE DISEASE VIRUS-BASED LIVE ATTENUATED VACCINE INOCULATION

4.1. Introduction

Lactic acid bacteria (LAB) are normal inhabitants of the gastrointestinal tract and classified as probiotic bacteria. Some of the benefits provided by LAB include the production of various nutrients for the host, prevention of infections caused by intestinal pathogens, and modulation of the normal immune response (Markowiak et al., 2017). Probiotics such as LAB can regulate inflammatory response by stimulating immune cells to produce cytokines. However, only a few published reports have demonstrated the benefits of probiotic bacteria with respect to the avian immune system. Furthermore, the effects of probiotics against viral infection in avian species have not yet been investigated. *Lactobacillus* spp. are the main type of LAB used in probiotic formulations because they possess potentially advantageous properties (Maragkoudakis et al., 2006). *Lactobacillus acidophilus* is considered a probiotic strain with anticancer and cholesterol-lowering properties and an antagonist against enteric pathogens. *L. acidophilus* has also been proven to elevate immunological activity by stimulating the innate and adaptive immune responses (Konstantinov et al., 2008; Zhang et al., 2008; Liu et al., 2010). Clinical trials involving the oral administration of *L. acidophilus* strain L-55 (LaL-55) showed that this strain effectively suppresses experimental allergic rhinitis (Gackowska et al. 2006) and experimental atopic dermatitis in mice (Zeuthen et al., 2006). *In vitro* studies have indicated that some specific strains of LAB are strong inducers of T helper type 1 (Th1) cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ (Sunada et al., 2008). One study has indicated that LaL-55 modulates the immune reaction of chickens inoculated with a Newcastle disease virus (NDV)-attenuated vaccine (Ho et al., 2020). Therefore, *L. acidophilus*, specifically LaL-55, was selected for this study to determine whether it may help protect chickens against infectious diseases by boosting immune function in the ileum, one of the gates for NDV. The data obtained from this study are also expected to provide useful information for future probiotic formulations that may help control the spread of infectious diseases in chickens.

Newcastle disease is one of the most important poultry viral diseases worldwide. NDV is classified into lentogenic (low virulence), mesogenic (moderate virulence), and velogenic (high virulence) strains according to the pathogenicity in chickens (Alexander, 1988). Live and inactivated NDV vaccines have been widely used. Live vaccines based on lentogenic strains are widely used because of their high efficacy and availability (Dimitrov et al., 2017). Inactivated oily vaccines are used for enhancing and sustaining immunity (Russell et al., 1997; Dimitrov et al.,

2017). The attenuated NDV vaccine used in this study was a lyophilized version of an attenuated NDV clone 30 injected into a growing chicken egg to evaluate the immune responses. Vaccines are commonly administered by the eye- or nose-drop method. Generally, cellular immunity is crucial in viral infection because the viral pathogenesis includes an intracellular phase. It is important to understand this response, and therefore estimation of the cellular immunity against viral infection such as NDV should be aligned with estimation of the humoral antibody response. Cell-mediated immune response is essential for virus clearance and may be a key player in vaccinal immunity to NDV (Russell et al., 1997). Lambrecht et al. (2004) reported the presence of antigen-specific chicken interferon (IFN)-γ production as an indicator of actively acquired immunity to NDV. Furthermore, cell-mediated immune response was detected in the spleens of chickens vaccinated twice or vaccinated and challenged with wild-type virus (Jeurissen et al., 2000).

IFNs play an essential role in innate immunity against viruses (Stetson et al., 2006). The crucial role of IFNs is the recognition of viral pathogen associated molecular patterns by cellular pattern recognition receptors (PRRs) (Majer et al., 2017). There are three major classes of PRRs associated with the activation of the IFN pathways. The first category of PRRs is the family of retinoic acid-inducible gene-I-like receptor including melanoma differentiation-associated gene 5 (MDA5). A second class of PRRs is the family of TLRs, such as TLR-3 and TLR7 (Thompson et al., 2011). The third category of PRRs is the family of DNA sensors (Servant et al., 2002). When these PRRs are activated, they can recruit specific adaptor proteins, such as myeloid differentiation primary response gene 88 (MyD88) or Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β. These signal pathways ultimately converge on the activation of IFN regulatory factors (IRFs). Activated IRFs translocate into the nucleus to regulate IFN expression. However, the immune status in the ileum of chickens after NDV vaccination has been unclear. The differences in immune status between the ileum and spleen in chickens inoculated with an attenuated NDV vaccine have also been unclear.

This study aimed to evaluate the beneficial effects of LaL-55 oral administration on the gene expression of cytokines, MDA5, and transcriptional factors (Blimp-1, IRF-3, IRF-7, MyD88, and STING) associated with antiviral immunity in the ileum and spleen of chicks inoculated with a live-attenuated NDV vaccine.

4.2. Materials and Methods

4.2.1. Animals

Eggs (White Leghorn) were purchased from Kui Poultry Co., Ltd. (Mihara, Hiroshima, Japan). Eggs were incubated at 37.7°C ± 1°C until hatching. After hatching, chicks were maintained in a coccidian-free room and provided feed and water *ad libitum*. The animals were housed at a constant temperature (27°C ± 1°C) with a 12 h dark/light cycle. All procedures were approved by the Animal Care and Use Committee of Okayama University (OKU-2020201) and conducted in compliance with the Policy on the Care and Use of the Laboratory Animals of Okayama University. Animal care and experiments were also carried out in accordance with the guidelines for animal experiments at Okayama University. The body weights of the chicks were measured daily during this experiment.

4.2.2. LaL-55 administration and NDV vaccine inoculation

Freeze-dried LaL-55 powder was prepared as reported previously (Sunada et al., 2007). Chicks (n = 24) were randomly divided into four groups and provided different concentrations of the freeze-dried LaL-55 (high-dose group, 0.5 mg/100 g body weight; middle-dose group, 0.15 mg/100 g body weight; low-dose group, 0.05 mg/100 g body weight; and control group, 0 mg/100 g body weight). Each volume of the freeze-dried LaL-55 powder was suspended in 1 mL distilled water and compulsively administered orally to chicks daily from 1 to 6 weeks of age. Identical volumes of distilled water were administered to chicks in the control group. The live-attenuated NDV vaccine (10 μL; ND clone 30; Intervet, Osaka, Japan) was dropped into the eye and nose at 2 weeks of age (primary inoculation). The same NDV vaccine dose was also administered to the same sites at 4 weeks of age in all treatment groups (secondary inoculation).

4.2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The mRNA levels for cytokines (IFN-α, IFN-β, IFN-γ, IFN-λ, and IL-1β), MDA5, and transcriptional factors (Blimp-1, IRF-3, IRF-7, MyD88, and STING) were determined using qRT-PCR. The sequences for the primer pairs used in this experiment are listed in table 4-1. The chicks were euthanized at the end of the experiment (4 and 6 weeks of age, 2 weeks after primary and secondary inoculation), and ileum and spleen tissues were collected. The tissues were preserved at -20°C until they were used for analyses RNA extraction was performed using TRIzol reagent

(Invitrogen, Carlsbad, CA, USA). The extracted RNA was treated with DNase I (Takara Bio, Otsu, Shiga, Japan), and the cDNA synthesis was carried out using oligo (dT_{18}).

Table 4- 1. Primer sets for quantitative RT-PCR.

	Primer sequence (5'-3')	Annealing temp. (°C)	Accession No.
IFN-α	Forward: GGGTACGACATCCTGTTGCTC Reverse: CGGCTGATCCGGTTGAGGAG	68.8	AB_021153
IFN-β	Forward: GCCACAGCCTCCTCAACCAGAT Reverse: CAACGTCCCAGGTACAAGCACT	60.0	AY_831397
IFN-γ	Forward: AAGTCAAAGCCGCTACATCAAAC Reverse: CTGGATTCTCAAGTCGTTCATCG	60.0	NM_205149.1
IFN-λ	Forward: GGAGGATGAAGGAGCAGTTTG Reverse: ACGGTGATGGTGAGGTCC	60.0	NM_001128496.1
IL-1β	Forward: GTACCGAGTACAACCCCTGC Reverse: AGCAACGGGACGGTAATGAA	60.0	XM_015297469.1
Blimp-1	Forward: GGCAGCCTGTCAGAATGGAAT Reverse: GCTCCTTCTTTGGGACGCTCT	60.0	XM_004940353.3
MDA5	Forward: GCAAAAACCAGCACTGAATGGG Reverse: CGTAAATGCTGTTCCACTAACGG	60.0	GU570144.1
IRF-3	Forward: ACCACATGCAGACAGACTGACACT Reverse: GGAGTGGATGCAAATGCTGCTCTT	60.0	AF268079.1
IRF-7	Forward: GCCTGAAGAAGTGCAAGGTC Reverse: CTCTGTGCAAAACACCCTGA	60.0	NM_205372.1
MyD88	Forward: AAGGTGTCGAGGATGGTGGTC Reverse: GGAATCAGCCGCTTGAGACGAG	60.0	NM_001030962.4
STING	Forward: GGTCCTACTACATCGGCTACCTGA Reverse: GGCCTGAGCTTGTTGTCCTTATCT	60.0	XM_015293528.2
β-actin	Forward: GAGAAATTGTGCGTGACATCA Reverse: CCTGAACCTCTCATTGCCA	60.0	NM_205518.1

Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories Inc.) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). The amplification of β-actin was used as an internal control. The samples were heated

at 95°C for 1 min and then subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing/elongation for 1 min at 60°C. Amplifications was performed for three independent samples per group, with triplicate reactions for each sample on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

4.2.4. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Data were statistically evaluated by two-way analysis of variance (ANOVA) with Tukey's test using IBM SPSS Statistics 20.0. Differences were considered significant at $p < 0.05$ (*) and $p < 0.01$ (**).

4.3. Results

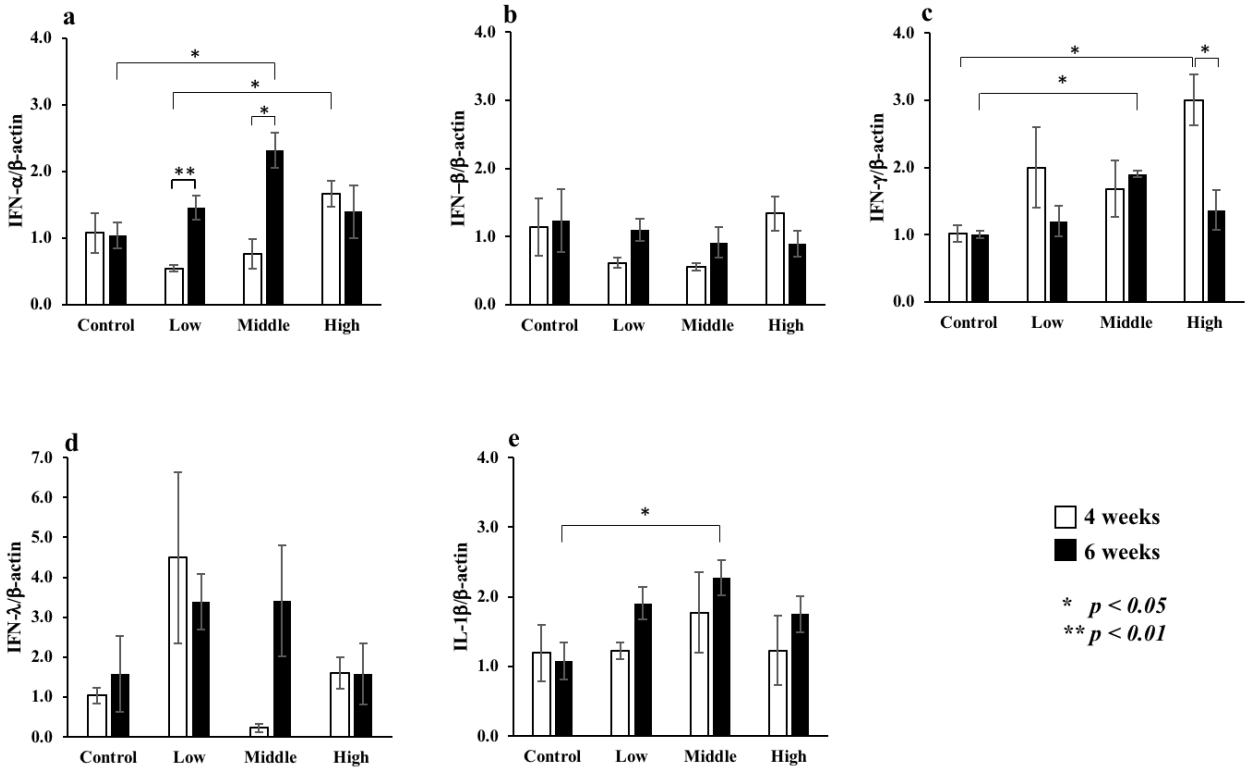


Figure 4- 1. mRNA expression levels of cytokines in the spleen of chickens inoculated with an NDV vaccine: (a) IFN-α, (b) IFN-β, (c) IFN-γ, (d) IFN-λ, and (e) IL-1β. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the 2−ΔΔCt method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0.

The results for the mRNA expression levels in the spleen are indicated in figure 4-1. The expression levels of the cytokines examined other than IFN-β and IFN-λ showed dose-dependent

increases at 6 weeks, reaching their peaks in the middle-dose LaL-55 group. The IFN-α and IFN-γ mRNA levels increased in a dose-dependent manner after LaL-55 administration (Figs. 4-1a & c). The IL-1β expression levels also increased in a dose-dependent manner after oral administration of LaL-55 (Fig. 4-1e). The IFN-β and IFN-λ mRNA levels did not change after LaL-55 administration (Figs. 4-1b & d). IFN-α mRNA expression was significantly higher in the low- and middle-dose groups at 6 weeks than in the spleen samples at 4 weeks ($p < 0.01$ and $p < 0.05$).

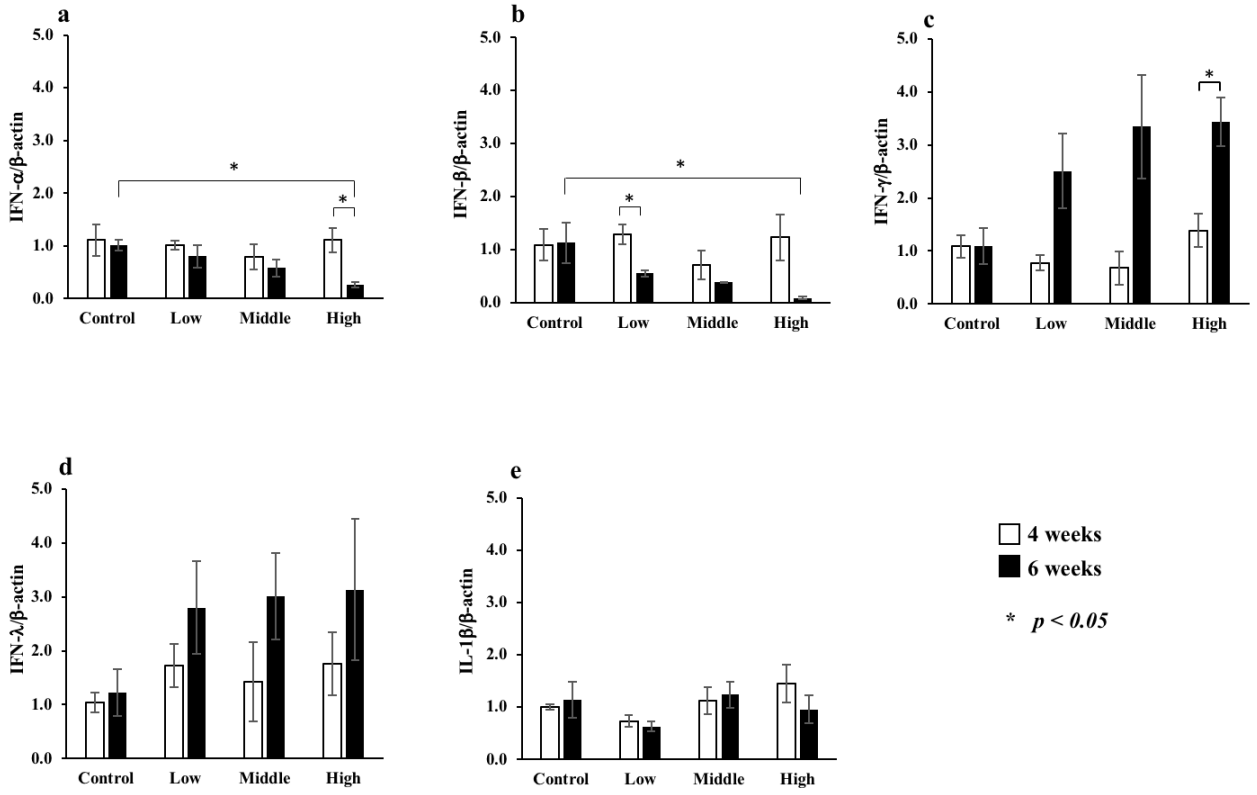


Figure 4- 2. mRNA expression levels of cytokines in the ileum of chickens inoculated with an NDV vaccine: (a) IFN-α, (b) IFN-β, (c) IFN-γ, (d) IFN-λ, and (e) IL-1β. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0.

The results for the cytokine mRNA expression levels in the ileum are indicated in Figure 2. The expression levels of all the cytokines examined in this study did not differ among the groups at 4 weeks. IFN-α and IFN-β mRNA expression decreased in a dose-dependent manner after oral administration of LaL-55 (Figs. 4-2a & b). In contrast, IFN-γ showed a dose-dependent increase after 6 weeks of oral administration of LaL-55, but the increase was not statistically significant. However, the IFN-γ mRNA expression level in the high-dose group was significantly higher at 6

weeks than at 4 weeks (Fig. 4-2c). The IFN-l expression levels at 6 weeks increased in a dose-dependent manner after the oral administration of LaL-55, but a statistical significance was not observed (Fig. 4-2d). The IL-1β mRNA level did not change after LaL-55 oral (Fig. 4-2e).

The mRNA expression levels of transcriptional factors in the spleen were also determined. The MDA5 expression levels in the spleen decreased in a dose-dependent manner after the oral administration of La-L55 (Fig. 4-3a). The IRF-3 and IRF-7 mRNA expression levels were significantly higher in the low-dose group than in the middle- and high-dose groups (Figs. 4-3b & c). The Blimp-1, STING, and MyD88 mRNA levels did not change in the spleen after LaL-55 administration (Figs. 4-3d, e & f).

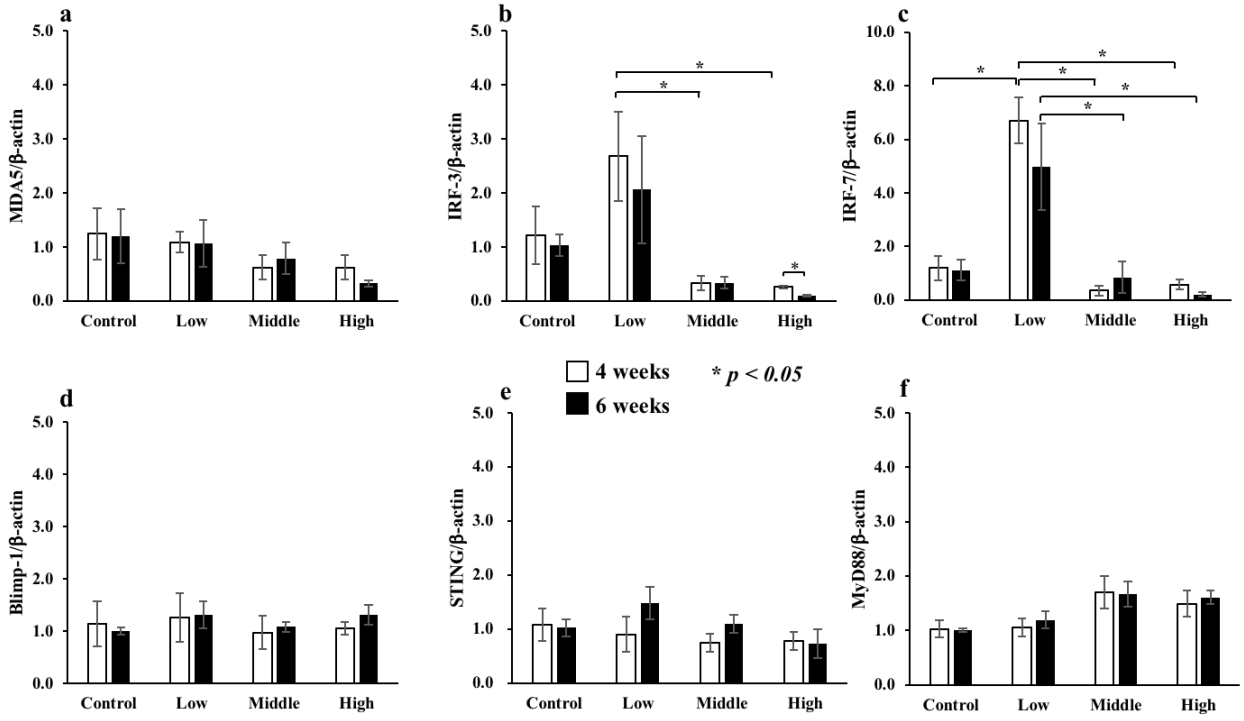


Figure 4- 3. mRNA expression levels of transcriptional factors in the spleen of chickens inoculated with an NDV vaccine: (a) MDA5, (b) IRF-3, (c) IRF-7, (d) Blimp-1, (e) STING, and (f) MyD88. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0.

Finally, the mRNA expression levels of transcriptional factors in the ileum were determined. The MDA5 expression level showed a dose-dependent increase at 4 weeks. However, the expression levels of MDA5 at 6 weeks were not different (Fig. 4-4a). The IRF-3 and MyD88 mRNA expression levels did not change in the ileum after LaL-55 administration (Figs. 4-4b & f).

The IRF-7 expression levels after 6 weeks of oral administration of LaL-55 showed dose-dependent increases and were higher than that of the high-dose group at 4 weeks (Fig. 4c; $p < 0.05$). The Blimp-1 and STING levels showed dose-dependent decreases at 4 weeks (Figs. 4-4d & e).

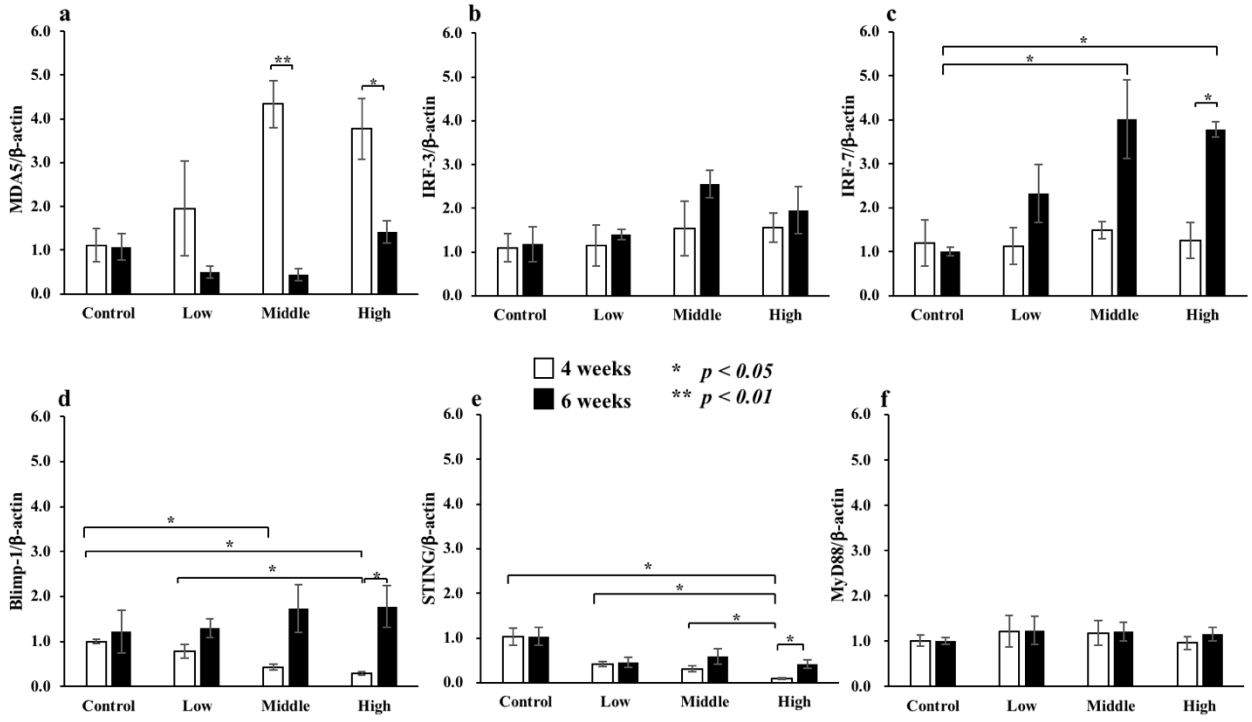


Figure 4- 4. mRNA expression levels of transcriptional factors in the ileum of chickens inoculated with an NDV vaccine: (a) MDA5, (b) IRF-3, (c) IRF-7, (d) Blimp-1, (e) STING, and (f) MyD88. Open columns represent 4-week-old chicks; filled columns represent 6-week-old chicks. Amplification was performed for three independent samples with triplicate reactions carried out for each sample. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the mean ± SEM and were analyzed by two-way ANOVA with Tukey's test using IBM SPSS Statistics 20.0.

4.4. Discussion

Infectious diseases caused by various pathogens, such as highly pathogenic strains of influenza and porcine fever, not only cause stunting of the livestock and poultry industries but can also fatal at times, causing enormous economic loss to these industries. Various strategies, including vaccination, have been used to combat pathogenic organisms. However, due to the excessive use of antibiotics among humans and livestock and changes in the environment, antibiotic-resistant organisms and some pathogens have posed a huge problem for public health. In parallel with the production of drugs and vaccines, probiotics are being considered as an additional effective method for disease prevention and treatment. Live bacteria supplements such as LAB

have recently become widely used, and probiotics have become an important target for providing health benefits in humans and animals. Recently, live and dead bacteria have been used to enhance the efficacy of vaccines. Probiotic bacteria have become a target of interest for promoting health benefits in humans and animals. LAB enhances vaccine efficacy by stimulating both the innate and adaptive immune responses (Maragkoudakis et al., 2006; Konstantinov et al., 2008; Zhang et al., 2008; Liu et al., 2010). This study focused on LaL-55 and determined its beneficial effects on chicks administered a vaccine. In a previous study, chickens that were supplemented with various *lactobacilli* showed enhanced IgM and IgG responses to soluble antigens (Koenen et al., 2004). In another study, the administration of probiotics containing *L. acidophilus* enhanced the serum IgA response to keyhole limpet hemocyanin (a highly immunogenic T-cell dependent antigen) (Huang et al., 2004). Hence, LaL-55 has been expected to promote beneficial effects on the immune system of the chicken. This study evaluated the effects of LaL-55 oral administration on the gene expression of cytokines and master immune switches in the ileum and spleen of chicks inoculated with an attenuated NDV vaccine.

IFNs are known to the essential factors in innate immune responses against virus infection (Jeurissen et al., 1996; Stetson & Medzhitov 2006). This study showed that IFN-γ mRNA expression in the spleen was upregulated after oral administration of the middle dose of LaL-55 at 2 weeks after secondary immunization with the NDV vaccine (Fig. 4-1c). In contrast, the IFN-β and IFN-λ mRNA levels did not change (Figs. 4-1b & d). The results also showed that dose-dependent upregulation of IFN-γ mRNA was observed in the ileum at 6 weeks (Fig. 2c). LAB stimulates IL-12 production by dendritic cells, increasing IFN-γ production and activation (Gackowska et al., 2006; Zeuthen et al., 2006; Fink et al., 2007). IFN-γ, which is produced by Th1, CTL, and NK cells, is involved in macrophage activation, Th1 differentiation, B cell differentiation, and NK cell activation. *In vitro* studies have indicated that *L. acidophilus* is a strong inducer of Th1 cytokines, including IL-12 and IFN-γ (Gackowska et al., 2006; Zeuthen et al., 2006). Administration of other LAB strains (*Lactobacillus brevis* and *L. acidophilus)* increases the production of Th1 cell-specific cytokines (IFN-γ and IL-12) (Segawa et al., 2008; Lee et al*.,* 2011). Data in a previous study have also showed that IFN-γ mRNA expression in splenic mononuclear cells was upregulated after the oral administration of the middle or high doses of LaL-55 at 2 weeks after secondary immunization with the NDV vaccine (Ho et al., 2020). Cytotoxic CD8 T cells are one of the major sources of IFN-γ production. However, the CD8 mRNA levels did not changed in

this study (data not shown). In this study, it is not clear which type of cells upregulated IFN-γ mRNA, as the spleen contains all previously mentioned types of cells. However, it is possible to assume that IFN-γ production is related to the enhanced activity of NK and Th1 cells. The elevation of IFN-γ mRNA levels has been shown in studies related to the effect of LAB on the Th1/Th2 balance.

The main function of the IFN-α is to alert the organism in the case of viral infection (McNab et al., 2015). IFN-α expression in spleen was enhanced at 6 weeks by the middle dose of LaL-55 administration in this study. However, our previous study showed that LaL-55 administration did not alter the expression levels of IFN-α in splenocytes at 6 weeks (Ho et al., 2020). The difference between this and the previous study is that the entire spleen was used in this study rather than just spleen monocytes. Since the entire spleen was used in this study, it is possible that there was a wide variety of IFN-α producing cells, including monocytes, present. IFN-α producing cells include a variety of cells such as macrophages, dendritic cells, lymphocytes, neutrophils, and fibroblasts. In the previous and this study, we did not examine granulocytes such as neutrophils. Our previous study indicated that total number of lymphocytes increased in the peripheral blood (Ho et al., 2020). It has also been reported that L. acidophilus promotes heterophil activation (Farnell et al., 2006). Therefore, regarding the usefulness of LaL-55 administration, it may be necessary to investigate IFN-α producing cells that may contain granulocytes such as heterophil other than monocytes in the future.

The mRNA levels of IFN-β were similar in all groups at the two-time points, demonstrating different effects of LaL-55 on different types of type I IFN. Comparing the IFN-α mRNA levels at the two time points, the expression of IFN-α was found to be low after the first vaccination with the low and medium doses of LaL-55. Initially, low levels developed in the 2 weeks after the first vaccination, but the level became even more pronounced at 2 weeks after the second vaccination. In the high-dose group, the expression level of IFN-α was higher than those in the other groups at 4 weeks. The difference compared with the low-dose group was significant. It is unknown why this changed at 6 weeks. Further research may be needed on the dose of LaL-55. On the other hand, IFN-α expression at 6 weeks was correlated with IL-1β (Figs. 4-1a & e). IL-1β is an inflammatory cytokine and is widely known as a potent modulator of IFN-α-induced antiviral gene expression (Ichikawa et al., 2002). IL-1β is also known to play an important role in both inflammation and viral pathogenesis (Ludwiczek et al., 2004; Sultan et al., 2017). Mingzhe et al. (2020) reported that

IL-1β may enhance the antiviral response of IFN-α by regulating the JAK-STAT signaling pathway. These facts may suggest that the expression of IFN-α in the spleen in the chicks administered both the low and medium doses of LaL-55 was controlled by IL-1β.

In the ileum at 6 weeks, IFN-α and IFN-β gene expression after NDV vaccination was inversely correlated with the amount of LaL-55 administrated at 6 weeks (Figs. 4-2a & b). The reduced expression of type I IFN observed as a results of the administration of high doses of LaL-55 may have occurred to avoid excessive downmodulation of the immune response in the negative feedback loop (Upasani et al., 2020). The results of this study may require further investigation of the appropriate doses and methods of administration for LaL-55.

Our results show that there are differences in the chicken immune response in the spleen and ileum to NDV vaccination in conjunction with frequent intake of an LaL-55 supplement. The most striking difference was the difference in the expression patterns of type I IFN in the spleen and ileum of the chicks administrated LaL-55 compared with the chicks in the control group. In the case of type II IFN, there was a significant upregulation in the spleen (Fig. 4-1c), and an upward trend in the ileum of chicks with LaL-55, but it was not significant (Fig. 4-2c). These reactions evoked by LaL-55 administration primarily involved the spleen, which is a secondary immune organ. In our previous research, we have shown that cytotoxic activity of NK cells was enhanced by LaL-55 administration (Ho et al., 2020). Since activated NK cells are also considered as type II IFN producing cells, it is possible that IFN was locally induced because of NK cell activation in the ileum. On the other hand, the expression of IFN-α and IFN-γ in the spleen may reflect the immune response as a secondary lymphoid organ. In other words, it may indicate an antiviral response as a result of antigen-presenting cells migrating to the spleen due to NDV infection of the ileum. As the spleen is one of the secondary lymphoid organs, boosting the immune defense in this organ is of great benefit for the animal to combat harmful agents. In fact, the enhancement of the cytokine expression in the chicken spleen by administration of a probiotic supplement has been reported previously (Jennifer et al., 2010; Wu et al., 2019).

In this study, oral administration of LaL-55 increased the levels of IRF-7 and IFN-γ mRNA in the ileum after the second NDV vaccination in a dose-dependent manner (Figs. 4-2c & 4-4c). IRF-7 expression in the spleen was significantly higher in the group using low-dose LaL-55 than in the middle- and high-dose groups at both 4 and 6 weeks. Similarly, IRF-3 expression was more prominent in the low-dose group compared with the middle- and high-dose groups, but the

differences were only significant at 4 weeks. The expression patterns of IRF-3 and IRF-7 in the spleen were not consistent with the expression patterns of IFN-α and IFN-γ; however, the expressions of these IFNs at 6 weeks were significantly increased in the middle-dose group compared with the control group (Figs. 4-1a & c). The expression of IFN-γ in the spleen at 4 weeks also increased in the high-dose group compared with control group, significantly (Fig. 4-1c). The IRF is composed of a growing family of related transcriptional proteins that were first identified as regulators of the IFN-α/β gene promoter and interferon-stimulated response components of several IFN-stimulating genes (Lin et al., 1998). IRF-3 and IRF-7 have additional self-suppressing motifs that are relaxed in response to double-stranded RNA induced by serine phosphorylation of TANK-binding kinase 1, but IRF-3 is limited in function without stimulation. IRF-7 is known to have additional elements that increase both basal and virus-inducing activity and is thought to be more indiscriminate with its binding motifs (Lin et al*.,* 2000). Further studies may be needed on the mechanism of regulation of gene expressions of antiviral factors after LaL-55 administration including post-transcriptional modification of these factors.

Recently, Carlin et al*.* (2017) reported that mice deficient in key transcriptional factors, including IRF-3 and IRF-7, remain resistant to severe dengue virus infection. Splenocytes from their gene knockout mice predominantly activate a transcriptional program compatible with the IL-12/IFN-γ/IRF-1 signaling pathway after dengue virus infection. These results may suggest that the oral administration of LaL-55 induces IFN-γ expression by an alternative pathway of IFN-γ production rather than the IRF-3, IRF-7, MDA5, and MyD88 signaling pathways and induces the local antiviral activity against NDV in the ileum of the chicken. Future studies are needed to investigate whether an alternative pathway of IFN-γ production is activated by LaL-55 administration.

Overall, LaL-55 enhances the immune response after the administration of a live-attenuated NDV vaccine by increasing the expression levels of IFN-γ but not type I IFN production in the ileum. Therefore, LaL-55 could be used as a tool to enhance the local immune response against viral infection in avian species. Additionally, LaL-55 enhances IFN-α and IFN-γ gene expression in the spleen after the administration of a live-attenuated NDV vaccine. However, it is unclear from the results of this study which signaling pathway is responsible for stimulation of the IFN-α and IFN-γ gene expression induced by LaL-55 administration. Further research is needed to make more effective use of LaL-55 in livestock production and human health.

CHAPTER V
GENERAL CONCLUSION

The modern commercial poultry industry, despite many advances that have been achieved to improve poultry health, continues to struggle with enteric disease, especially avian coccidiosis. Chickens that were raised at high density on an industrial scale are highly susceptible to this type of disease. Wildly used methods for controlling enteric pathogens include anticoccidial chemotherapy and vaccination. Although using the anticoccidial drug for parasitic control in integrated broiler production is common, consumers prefer "drug-free" products in the context of drug resistance, leading to the search for alternative control measures. On the other hand, vaccination has been used for decades by the industry but to date, the practice has received only limited acceptance in vertically integrated chicken production.

The research described in this thesis had focused on the mechanisms of intestinal barrier disruption and the beneficial effect of LaL-55 probiotic against coccidiosis. Furthermore, the investigation on the augmentation of live-attenuated NDV vaccination efficacy by the use of probiotic bacteria was also reported.

The impairment of the intestinal barrier function is related to the pathogenesis of many intestinal inflammatory syndromes. Thus, investigations aimed at understanding the mechanisms of intestinal barrier function are critical to developing preventative clinical strategies to reduce the incidence or the severity of these devastating diseases. Molecular mechanisms of intestinal barrier disruption by *Eimeria tenella* infection have not been fully elucidated by previous studies, therefore our research has focused on the relationship between clinical signs and intestinal conditions in chicken with *E. tenella* infection. Results from Chapters II of this thesis suggest that the typical clinical signs such as diarrhea and bloody feces in *E. tenella* infection are not related to detachment of the epithelial layer, but the disruption of the intestinal epithelial barrier via changes in gene expressions of related proteins in the chick cecum. To be specific, gene expressions of Claudin-3, Occludin, Zonula occludens-1, and E-cadherin were decreased, while the gene expression of Claudin-2 was increased upon infection, causing the impairment of gut barrier function and leading to gut leakage. We also found that transcript levels of pro-inflammatory cytokines namely IL-22 and IL-1β were increased in response to *E. tenella* infection. Further studies on the mechanisms of inflammation-dependent alteration of junctional genes would be of great benefit to the development of therapeutics to improve mucosal healing and barrier function against Coccidiosis by E. *tenella*.

Probiotic bacteria have been widely used for promoting health benefits in humans and animals. In Chapter III, the observation regarding the effects of LaL-55 administration on chicken

infected with *E. tenella* has been reported. Specifically, we found that the supplement of LaL-55 bacteria was beneficial towards the intestinal integrity of chicks. Our results suggest that LaL-55 administration did not trigger a statistically significant recovery in the impaired intestinal barrier by *E. tenella* infection, but it can help to mitigate the clinical signs and reduce the number of oocysts shedding and partially reverse the downregulation of CLDN-3, OLDN, ZO-1 and E-cad expression upon *E. tenella* infection in order to maintain the intestinal barrier integrity.

LAB can enhance vaccine efficacy by stimulating both innate and adaptive immune responses. The positive impact of probiotic administration on vaccination has been observed through the experiment of Chapters IV. Indeed, the data of this chapter indicate that LaL-55 enhances the chick immune response during NDV vaccinations, albeit different mechanisms occurred between the spleen and ileum. In the spleen, the transcript level of IFN-α in chicks taking certain doses of LaL-55 was significantly higher after the second vaccination but unchanged in chicks without taking probiotic. Moreover, gene expressions of IFN regulatory factors (IFR)-3 and IRF-7 were significantly higher when taking low dose of LaL-55, implicating the improvement of chick immune response in the spleen. Regarding the ileum, the mRNA levels of melanoma differentiation-associated protein 5, IFN-γ, and IRF-7 were increased if LaL-55 was supplemented, revealing the advantageous effect of LaL55 administration towards chick immune response at the ileum.

Taken together, the data presented in Chapters II, III, and IV of this thesis suggest that probiotic administration can improve intestinal health during periods of clinical coccidiosis invasion and enhances the local immune response against viral infection in avians under NDV vaccination. The data obtained in this study have provided useful information for future probiotic formulations, which may help to control the spread of infectious diseases in chickens. More research needs to be performed to validate and extend these findings to assist with improving probiotic efficacy in the future.

REFERENCE

Abdelrazek, Y., Desouky, M., Khaled, Sultan., Nagwa, M., Elhawary, Ammar, N., 2020. Levamisole Hydrochloride as Immunostimulant Drug Synergies the Effect of *Eimeria tenella* Lab- made Vaccine Experimental Trial. Asian J of Animal Sciences, 14, 54-60.

Ahmad, R., Chaturvedi, R., Olivares-Villagómez, D., Habib, T., Asim, M., Shivesh, P., Polk, D. B., Wilson, K. T., Washington, M. K., Kaer, L. van, Dhawan, P., & Singh, A. B., 2014. Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. Mucosal Immunology, 7, 1340–1353.

Ahmed, S.T., Islam, M.M., Mun, H.S., Sim, H.J., Kim, Y.J., Yang, C.J., 2014. Effects of Bacillus amyloliquefaciens as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poultry Science, 93, 1963–1971.

Alexander, D.J., 1988. Newcastle Disease Virus-an Avian Paramyxovirus. Springer, Boston, 11–22.

Amasheh, S., Fromm, M., Günzel, D., 2011. Claudins of intestine and nephron - a correlation of molecular tight junction structure and barrier function. Acta Physiologica, 201, 133–140.

Artis, D., 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Reviews Immunology, 8, 411–420.

Awad, W.A., Ghareeb, K., Abdel-Raheem, S., Böhm, J., 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry Science, 88, 49–55.

Awad, W.A., Ghareeb, K., Böhm, J., 2010. Effect of addition of a probiotic micro-organism to broiler diet on intestinal mucosal architecture and electrophysiological parameters. Journal of Animal Physiology and Animal Nutrition, 94, 486–494.

Awad, W. A., Hess, C., & Hess, M., 2017. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins, 9.

Barrios M.A., M. Da Costa, E. Kimminau, L. Fuller, S. Clark, G. Pesti, R. Beckstead, 2017. Relationship between broiler body weights, Eimeria maxima gross lesion scores, and microscores in three anticoccidial sensitivity tests. Avian Dis., 61, 237-241.

Barber, M., Aldridge, J., Webster, R., Magor, K., 2010. Association of RIG-I with innate immunity of ducks to influenza. Proceedings of the National Academy of Sciences of the USA, 107, 5913–5918.

Bai, S.P., Wu, A.M., Ding, X.M., Lei, Y., Bai, J., Zhang, K.Y., Chio, J.S., 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poultry Science, 92, 663–670.

Berkes, J., Viswanathan, V.K., Savkovic, S.D., Hecht, G., 2003. Intestinal epithelial responses to enteric pathogens: Effects on the tight junction barrier, ion transport, and inflammation. Gut, 52, 439–451.

Beutel, O., Maraspini, R., Pombo-García, K., Martin-Lemaitre, C., Honigmann, A., 2019. Phase Separation of Zonula Occludens Proteins Drives Formation of Tight Junctions. Cell, 179, 923–936.

Bjerknes, M., Cheng, H., 2005. Gastrointestinal stem cells. II. Intestinal stem cells. American Journal of Physiology - Gastrointestinal and Liver Physiology, 289, 381–387.

Brisbin, J.T., Gong, J., Orouji, S., Esufali, J., Mallick, A.I., Parvizi, P., Shewen, P.E., Sharif, S., 2011. Oral treatment of chickens with lactobacilli influences elicitation of immune responses. Clinical and Vaccine Immunology, 18, 1447–1455.

Broom, L.J., Kogut, M.H., 2018. Gut immunity: Its development and reasons and opportunities for modulation in monogastric production animals. Animal Health Research Reviews, 19, 46–52.

Burrell, A., Tomley, F. M., Vaughan, S., & Hernandez, V., 2019. Life cycle stages, specific organelles, and invasion mechanisms of Eimeria species. Parasitology, 147, 263-278.

Byrnes, S., Eaton, R., Kogut, M., 1993. In vitro interleukin-1 and tumor necrosis factor-alpha production by macrophages from chickens infected with either Eimeria maxima or Eimeria tenella. International Journal for Parasitology, 23, 639–645.

Cao, G.T., Zeng, X.F., Chen, A.G., Zhou, L., Zhang, L., Xiao, Y.P., Yang, C.M., 2013. Effects of a probiotic, *Enterococcus faecium*, on growth performance, intestinal morphology, immune response, and cecal microflora in broiler chickens challenged with *E. coli* K88. Poultry Sci., 92, 2949–2955.

Cani, P. D., Possemiers, S., van de Wiele, T., Guiot, Y., Everard, A., Rottier, O., Geurts, L., Naslain, D., Neyrinck, A., Lambert, D. M., Muccioli, G. G., & Delzenne, N. M., 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut, 58, 1091–1103.

Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. Biochimica et Biophysica Acta – Biomembranes, 1788, 864–871.

Chapman, H.D., 2014. Milestones in avian coccidiosis research: A review. Poultry Sci., 93, 501–511.

Carlin, A.F., Plummer, E.M., Vizcarra, Sheets, N., Joo, Y., Tang, W., Day, J., Greenbaum, J., Glass, C., Diamond, M., Shresta, S., 2017. An IRF-3-, IRF-5-, and IRF-7-Independent Pathway of Dengue

Viral Resistance Utilizes IRF-1 to Stimulate Type I and II Interferon Responses. Cell Reports, 21, 1600–1612.

Chapman H.D., J.R. Barta, D. Blake, A. Gruber, M. Jenkins, N.C. Smith, X. Suo, F.M. Tomley, 2013. A selective review of advances in coccidiosis research. Advances in Parasitology. D. Rollinson, edElsevier, London, UK, 93–171.

Cheng, Y., Chen, Y., Li, X., Yang, W., Wen, C., Kang, Y., Wang, A., Zhou, Y., 2017. Effects of synbiotic supplementation on growth performance, carcass characteristics, meat quality and muscular antioxidant capacity and mineral contents in broilers. Journal of the Science of Food and Agriculture, 97, 3699–3705.

Chen, J., Tellez, G., Richards, J.D., & Escobar, J., 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. Front. Vet. Sci, 2,14.

Chelakkot, C., Ghim, J., & Ryu, S. H., 2018. Mechanisms regulating intestinal barrier integrity and its pathological implications. Experimental and Molecular Medicine, 50.

Chida, R. U., An, J. H., & Soda, H. I., 2009. Effect of Capsaicin on the Tight Junctional Permeability of the Human Intestinal Cells. Journal of Arid Land Studies, 19, 89–92.

Chitapanarux, I., Chitapanarux, T., Traisathit, P., Kudumpee, S., Tharavichitkul, E., Lorvidhaya, V., 2010. Randomized controlled trial of live *L. acidophilus* plus *B. bifidum* in prophylaxis of diarrhea during radiotherapy in cervical cancer patients. Radiation Oncology 5, 1–6.

Chih-Yuan C., Li-Tsen C., Yue-Cheng C., Chun-Li L., Yi-Yang L. and Hau-Yang T., 2016. Use of a probiotic to ameliorate the growth rate and the inflammation of broiler chickens caused by *Eimeria tenella* infection. J. Anim. Res. Nutr, 1, 1-7.

Chow, Y. P., Wan, K. L., Blake, D. P., Tomley, F., & Nathan, S., 2011. Immunogenic *Eimeria tenella* glycosylphosphatidylinositol-anchored surface antigens (SAGs) induce inflammatory responses in avian macrophages. PLoS ONE, 6.

Cunningham, K.E., Turner, J.R., 2012. Myosin light chain kinase: Pulling the strings of epithelial tight junction function. Annals of the New York Academy of Sciences, 1258, 34–42.

Dalloul, R.A., Lillehoj, H.S., 2006. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Review of Vaccines, 5, 143–163.

Dalloul, R. A., Bliss, T. W., Hong, Y.-H., Ben-Chouikha, I., Park, D. W., Keeler, C. L., & Lillehoj, H. S., 2007. Unique responses of the avian macrophage to different species of *Eimeria*. Molecular Immunology, 44, 558–566.

Dalloul, R.A., Lillehoj, H.S., 2005. Recent advances in immunomodulation and vaccination strategies against coccidiosis. Avian Diseases, 49, 1-8.

Dalloul, R.A., Lillehoj, H.S., Shellem, T.A., Doerr, J.A., 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poultry Sciences, 82, 62-66.

Delva, E., Tucker, D.K., Kowalczyk, A.P., 2009. The Desmosome. Cold Spring Harbor perspectives in biology 1, a002543.

Dimitrov, K.M., Afonso, C.L., Yu, Q., Miller, P.J., 2017. Newcastle disease vaccines-A solved problem or a continuous challenge?. Veterinary Microbiology, 206, 126–136.

Dong, H., Wang, Y., Han, H., Li, T., Zhao, Q., Zhu, S., Li, L., Wu, Y., Huang, B., 2014. Identification and characterization of an Eimeria-conserved protein in *E. tenella*. Parasitol Res, 113, 735–745.

Dong, X., Abdelnabi, G.H., Lee, S.H., Li, G., Jin, H., Lillehoj, H.S., Suo, X., 2011. Enhanced egress of intracellular *Eimeria tenella* sporozoites by splenic lymphocytes from coccidian-infected chickens. Infection and immunity, 79, 3465–3470.

Dowland, S.N., Madawala, R.J., Lindsay, L.A., Murphy, 2016. The adherens junction is lost during normal pregnancy but not during ovarian hyperstimulated pregnancy. Acta His, 118, 137–143.

Du E.C., Wang W.W., Gan L.P., Li Z., Guo S.S., Guo Y.M., 2016. Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of broiler chickens challenged with Clostridium Perfringens. J Anim Sci Biotechnololy, 7, 19.

El-Sherry, S., M.E. Ogedengbe, M.A. Hafeez, M. Sayf-Al-Din, N. Gad, J.R. Barta, 2019. Cecal coccidiosis in turkeys: comparative biology of *Eimeria* species in the lower intestinal tract of turkeys using genetically typed, single oocyst–derived lines. Parasitol. Res., 118 , 583-598.

El-Ashram, S., Aboelhadid, S.M., Arafa, W.M., Gadelhaq, S.M., Abdel-Razik, A.R.H., 2019. Protective potential of diclazuril-treated oocysts against coccidiosis in layer chicks. Vet. Para., 273, 105–111.

Erben, U., Loddenkemper, C., Doerfel, K., Spieckermann, S., Haller, D., Heimesaat, M. M., Zeitz, M., Siegmund, B., & Kühl, A. A., 2014. A guide to histomorphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol, 7, 4557–4576.

Ewaschuk, J., Diaz, H., Meddings, L., Diederichs, Dmytrash, A., Backer, J., Looijer-van Langen, M., Madsen, K., 2008. Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. American journal of physiology. Gastrointestinal and liver physiology, 295.

Estela Quiroz-Castaneda, R., & Dantan-Gonzalez, E., 2015. Control of Avian Coccidiosis: Future and Present Natural Alternatives. BioMed Research International, 1–11.

Eyerich, K., Dimartino, V., & Cavani, A., 2017. IL-17 and IL-22 in immunity: Driving protection and pathology. European Journal of Immunology, 47, 607–614.

Farnell, M.B., Donoghue, A.M., Solis de los Santos F., Blore, P.J., Hargis, P.M., Tellez, G., Donoghue D.J., 2006. Upregulation of Oxidative Burst and Degranulation in Chicken Heterophils Stimulated with Probiotic Bacteria. Poultry Science, 85, 1900-1906.

Fatoba, A.J., Adeleke, M.A., 2018. Diagnosis and control of chicken coccidiosis: a recent update. Journal of Parasitic Diseases, 42, 483–493.

Fernando, M.A., Al-Attar, M.A., 1983. Invasion of Chicken Caecal and Intestinal Lamina Propria by Crypt Epithelial cells Infected with Coccidia. Parasitology, 86, 391–398.

Ferreri, D.M., Vincent, P.A., 2008. Signaling To and Through The Endothelial Adherens Junction. In: Cell Junctions: Adhesion, Development, and Disease. Wiley-VCH Verlag GmbH & Co., 169–195.

Ferrier, L., Mazelin, L., Cenac, N., Desreumaux, P., Janin, A., Emilie, D., Colombel, J.F., Garcia-Villar, R., Fioramonti, J., & Bueno, L., 2003. Stress-induced disruption of colonic epithelial barrier: role of interferon-gamma and myosin light chain kinase in mice. Gastroenterology Sep, 125, 795-804.

Food and Agriculture Organization of the United Nations (FA), Meat Market Review, Emerging trends and outlook, December 2020

Fink, L.N., Zeuthen, L.H., Ferlazzo, G., Frøkiær, H., 2007. Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. FEMS Immunology and Medical Microbiology, 51, 535–546.

Forte, C., Acuti, G., Manuali, E., Casagrande Proietti, P., Pavone, S., Trabalza-Marinucci, M., Moscati, L., Onofri, A., Lorenzetti, C., Franciosini, M.P., 2016. Effects of two different probiotics on microflora, morphology, and morphometry of gut in organic laying hens. Poultry Science, 95, 2528–2535.

France, M.M., Turner, J.R., 2017. The mucosal barrier at a glance. Journal of Cell Sci., 130, 307–314.

Furuse, M., Furuse, K., Sasaki, H., Tsukita, S., 2001. Conversion of Zonulae Occludentes from Tight to Leaky Strand Type by Introducing Claudin-2 into Madin-Darby Canine Kidney I Cells. The Journal of Cell Biology, 153, 263–272.

Fuller R., 1989. Probiotics in man and animals. *J. Appl. Bacte- riol.* 66, 365-378.

Fuller R., 2001. The chicken gut microflora and probiotic supplements. Poultry Sciences, 38: 189–196.

Gackowska, L., Michalkiewicz, J., Krotkiewski, M., Helmin-Basa, A., Kubiszewska, I., Dzierzanowska, D., 2006. Combined effect of different lactic acid bacteria strains on the mode of cytokines pattern

expression in human peripheral blood mononuclear cells. Article in Journal of physiology and pharmacology: an official journal of the Polish Physiological Society, 13–21.

Gadde, U., Kim, W.H., Oh, S.T., Lillehoj, H.S., 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: A review. Ani. Health Research Reviews, 18, 26–45.

Garcia-Hernandez, V., Quiros, M., Nusrat, A., 2017. Intestinal epithelial claudins: Expression and regulation in homeostasis and inflammation. Annals of the NY Academy of Sci., 1397, 66–79.

Gaffen, S. L., 2009. Structure and signaling in the IL-17 receptor family. Nature Re. of Im., 9, 556–567.

Ghareeb K., W.A. Awad, M. Mohnl, R. Porta, M. Biarnés, J. Böhm, G.Schatzmay, 2012. Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of Campylobacter jejuni in broiler chickens. Poultry Science, 91, 1825-1832.

González-Mariscal, L., Betanzos, A., Nava, P., Jaramillo, B.E., 2003. Tight junction proteins. Biophysics & Molecular Biology, 81, 1–44.

Graham, W.V., Wang, F., Clayburgh, D.R., Cheng, J.X., Yoon, B., Wang, Y., Lin, A., Turner, J.R., 2006. Tumor necrosis factor-induced long myosin light chain kinase transcription is regulated by differentiation-dependent signaling events: Characterization of the human long myosin light chain kinase promoter. Journal of Biological Chemistry, 281, 26205–26215.

Groschwitz, K.R., Hogan, S.P., 2009. Intestinal barrier function: Molecular regulation and disease pathogenesis. Journal of Allergy and Clinical Immunology,124, 3-20.

Guillemot, L., Paschoud, S., Jond, L., Foglia, A., Citi, S., 2008. Paracingulin regulates the activity of Rac1 and RhoA GTPases by recruiting Tiam1 and GEF-H1 to epithelial junctions. Molecular Biology of the Cell, 19, 4442–4453.

Guo, F., Li, P., French, J.B., Mao, Z., Zhao, H., Li, S., Nama, N., Fick, J.R., Benkovic, S.J., Huang, T.J., 2015. Controlling cell-cell interactions using surface acoustic waves. Proceedings of the National Academy of Sciences of the United States of America, 112, 43–48.

Gyorke, A., Pop, L., & Cozma, V., 2013. Prevalence and distribution of Eimeria species in broiler chicken farms of different capacities. Parasite, 20.

Haworth, K. E., El-Hanfy, A., Prayag, S., Healy, C., Dietrich, S., & Sharpe, P., 2005. Expression of Claudin-3 during chick development. Gene Expression Patterns, 6, 40–44.

Hafez HM, 2010. Poultry health-looking ahead to 2034. World Poult. 25:16–7.

Harhaj, N.S., Antonetti, D.A., 2004. Regulation of tight junctions and loss of barrier function in pathophysiology. International Journal of Biochemistry and Cell Biology, 36, 1206–1237.

Higgins J.P., S.E. Higgins, J.L. Vicente, A.D. Wolfenden, G. Tellez, B.M.Hargis, 2007. Temporal effects of LAB probiotic culture on *Salmonella* in neonatal broilers. Poultry Science, 86, 1662-1666.

Ho, D.T., Hatabu, T., Sunada, Y., Kondo, Y., 2020. Oral administration of the probiotic bacterium *L. acidophilus* strain L-55 modulates the immunological parameters of the laying hen inoculated with a Newcastle disease virus-based live attenuated vaccine. Bio. of Micro. Fo. and He., 39, 117–122.

Ho, T. D., Pham, H. S. H., Aota, W., Matsubayashi, M., Tsuji, N., Hatabu, T., 2021. Reduction of macrophages by carrageenan decreases oocyst output and modifies local immune reaction in chick cecum with *Eimeria tenella*. Research in Veterinary Science, 139, 59-66.

Hollander, D., 1999. Intestinal permeability, leaky gut, and intestinal disorders. Current gastroenterology reports, 1, 410–416.

Hollemans, M.S., van Baal, de Vries, G., Kemp, B., Lammers, A., de Vries, S., 2020. Intestinal epithelium integrity after delayed onset of nutrition in broiler chickens. Poultry Sci. 99, 6818–6827.

Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006a. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Veterinary Immunology and Immunopathology, 114, 209–223.

Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006b. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following Eimeria maxima infection of chickens. Veterinary Immunology and Immunopathology, 114, 259–272.

Hossain, Z., & Hirata, T., 2008. Molecular mechanism of intestinal permeability: interaction at tight junctions. Molecular BioSystems, 4, 1181–1185.

Hu, Y.J., Wang, Y.D., Tan, F.Q., Yang, W.X., 2013. Regulation of paracellular permeability: Factors and mechanisms. Molecular Biology Reports, 40, 6123–6142.

Huang, G., Tang, X., Bi, F., Hao, Z., Han, Z., Suo, J., Zhang, S., Wang, S., Duan, C., Yu, Z., Yu, F., Yu, Y., Lv, Y., Suo, X., Liu, X., 2018. Eimeria tenella infection perturbs the chicken gut microbiota from the onset of oocyst shedding. Veterinary Parasitology, 258, 30–37.

Huang, M. K., Y. J. Choi, R. Houde, J. W. Lee, B. Lee, and X. Zhao., 2004. Effects of Lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. Poultry Science, 83: 788–795.

Hutt, P., Shchepetova, J., Loivukene, K., Kullisaar, T., Mikelsaar, M., 2006. Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. Journal of Applied Microbiology, 100, 1324–1332.

Huyghebaert, G., Ducatelle, R., van Immerseel, F., 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal, 187, 182–188.

Ichikawa T, Nakao K, Nakata K, Yamashita M, Hamasaki K, Shigeno M, Abiru S, Ishikawa H, Ishii N, and Eguchi K., 2002. Involvement of IL-1beta and IL-10 in IFN-alpha-mediated antiviral gene induction in human hepatoma cells. Biochem. Biophys. Res. Commun., 294: 414-422.

Ikenouchi, J., Umeda, K., Tsukita, S., Furuse, & Tsukita, 2007. Requirement of ZO-1 for the formation of belt-like adherens junctions during epithelial cell polarization. J. of Cell Bi., 176, 779–786.

Iwakura, Y., Ishigame, H., Saijo, S., & Nakase, S., 2011. Functional specialization of interleukin-17 family members. Immunity, 34, 149–162.

Inagaki-Ohara, K., Dewi, F.N., Hisaeda, H., Smith, A.L., Jimi, F., Miyahira, M., Abdel-Aleem, A.S.F., Horii, Y., Nawa, Y., 2006. Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against Eimeria vermiformis infection. Infection and Immunity, 74, 5292–5301.

Jeurissen, S.H.M., Boonstra-Blom, A.G., Al-Garib, S.O., Hartog, L., Koch, G., 2000. Defence mechanisms against viral infection in poultry: A review. Veterinary Quarterly, 22, 204–208.

Jefferies CA. 2019. Regulating IRFs in IFN driven disease. Frontiers in Immunology, 10, 325.

Jennifer TB, Joshua G, Payvand P, Shayan S., 2010. Effects of *Lactobacilli* on Cytokine Expression by Chicken Spleen and Cecal Tonsil Cells. Clin Vaccine Immunol., 17: 1337–1343.

Jordan, D., Caldwell, Klein, Coppedge, Pohl , Fitz-Coy & Lee, 2011. *E. tenella* oocyst shedding and output in cecal or fecal contents following challenge in broilers. Poultry Science, 90, 990–995.

Kaneko, N., Kurata, M., Yamamoto, T., Morikawa, S., & Masumoto, J., 2019. The role of interleukin-1 in general pathology. Inflammation and Regeneration, 39, 1–16.

Kumar, A., Chatterjee, I., Anbazhagan, A. N., Jayawardena, D., Priyamvada, S., Alrefai, W. A., Sun, J., Borthakur, A., & Dudeja, P. K., 2018. *C. parvum* disrupts intestinal epithelial barrier function via altering expression of key tight junction and adherens junction proteins. Cellular Micro., 20, 1–13.

Kabir S.M.L., Rahman M.M., Rahman M.B., Hosain M.Z., Akand M.S.I. and Das S.K., 2005. Viability of probiotics in balanc- ing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. Biotechnology, 4, 325-330.

Kagnoff, M.F., 2014. The intestinal epithelium is an integral component of a communications network. Journal of Clinical Investigation, 124, 2841–2843.

Kale, G., Naren, A.P., Sheth, P., Rao, R.K., 2003. Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3. Bio. and Biophysi. Res. Communications, 302, 324–329.

Kalavathy R., Abdullah N., Jalaludin S. and HO Y.W., 2003. Effects of *Lactobacillus* cultures on growth performance, ab- dominal fat deposition, serum lipids and weight of organs of broiler chickens. *Br. Poult. Sci.,* 44, 139-144.

Kang, E.J., Kim, S.Y., Hwang, I.H., Ji, Y.J., 2013. The effect of probiotics on prevention of common cold: A meta-analysis of randomized controlled trial studies. Korean Journal of Family Medicine, 34, 2–10.

Karimi Kivi R., Dadashbeiki M. and Seidavi A.R., 2015. Growth, body characteristics and blood parameters of ostrich chickens receiving commercial probiotics. *Span J. Agric. Res*. 13, 1-11.

Karpala AJ, Stewart C, McKay J, Lowenthal J, Bean A., 2011. Characterization of Chicken MDA5 Activity: Regulation of IFN-β in the Absence of RIG-I Functionality. The Journal of Immunology, 186, 5397–5405.

Kawasaki, K., Hayashi, Y., Nishida, Y., Miki, A., Itoh, H., 1998. Developmental expression of the tight junction protein, occludin, in the gastrointestinal tract of the chick embryo. Histochem Cell Biol, 109, 19–24.

Kazemi, S.A., Ahmadi, H., Karimi Torshizi, M.A., 2019. Evaluating two multistrain probiotics on growth performance, intestinal morphology, lipid oxidation and ileal microflora in chickens. Journal of Animal Physiology and Animal Nutrition, 103, 1399–1407.

Kim, W.H., Chaudhari, A.A., Lillehoj, H.S., 2019. Involvement of T Cell Immunity in Avian Coccidiosis. Frontiers in Immunology, 10, 1–8.

Kindon, H., Pothoulakis, C., Thim, L., Lynch-Devaney, K., Podolsky, D.K., 1995. Trefoil peptide protection of intestinal epithelial barrier function: Cooperative interaction with mucin glycoprotein. Gastroenterology, 109, 516–523.

King, S., Glanville, J., Sanders, M.E., Fitzgerald, A., Varley, D., 2014. Effectiveness of probiotics on the duration of illness in healthy children and adults who develop common acute respiratory infectious conditions: A systematic review and meta-analysis. British J of Nutrition, 112, 41-54.

Kizerwetter-Swida M, Binek M., 2005. Selection of potentially probiotic *Lactobacillus* strains towards their inhibitory activity against poultry enteropathogenic bacteria. Polish J. Micro., 54, 287–294.

Konstantinov, S.R., Smidt, H., de Vos, W.M., Bruijns, S.C.M., Singh, S.K., Valence, F., Molle, D., Lortal, S., Altermann, E., Klaenhammer, T.R., van Kooyk, Y., 2008. S layer protein A of Lactobacillus acidophilus NCFM regulates immature dendritic cell and T cell functions. Proceedings of the National Academy of Sciences of the USA, 105, 19474–19479.

Koenen, M. E., J. Kramer, R. van der Hulst, L. Heres, S. H. M Jeurissen,and W. J. A. Boersma., 2004. Immunomodulation by probiotic Lactobacilli in layer- and meat-type chickens. Br. Poultry Science, 45, 355–366.

Kuttappan, V.A., Vicuña, E.A., Latorre, J.D., Wolfenden, A.D., Téllez, G.I., Hargis, B.M., Bielke, L.R., 2015. Evaluation of gastrointestinal leakage in multiple enteric inflammation models in chickens. Frontiers in Veterinary Science, 2, 66.

Lan Y., Xun S., Tamminga S., Williams B.A., Verstegen M.W. and Erdi G., 2004. Real-time PCR detection of lactic acid bacteria in cecal contents of *Eimeria tenella*-infected broilers fed soybean oligosaccharides and soluble soybean polysaccha- rides. Poultry Science, 83, 1696-1702.

Lan, L., Zuo, B., Ding, H., Huang, Y., Chen, X., Du, A., 2016. Anticoccidial evaluation of a traditional Chinese medicine Brucea javanica in broilers, Poultry Science, 95, 811-818.

Lal, K., Bromley, E., Oakes, R., Prieto, J.H., Sanderson, S.J., Kurian, D., Hunt, L., Yates, J.R., Wastling, J.M., Sinden, R.E., Tomley, F.M., 2009. Proteomic comparison of four Eimeria tenella life-cycle stages: Unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. Proteomics, 9, 4566–4576.

Laurent, F., Mancassola, R., Lacroix, S., Menezes, R., & Naciri, M., 2001. Analysis of Chicken Mucosal Immune Response to *Eimeria tenella* and *Eimeria maxima* Infection by Quantitative Reverse Transcription-PCR. Infection and Immunity, 69, 2527–2534.

Lambrecht, B., Gonze, M., Meulemans, G., van denBerg, T.P., 2004. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon γ in response to mitogen and recall Newcastle disease viral antigen stimulation. Avian Pathology, 33, 343-350.

Lechuga, S., & Ivanov, A. I., 2017. Disruption of the epithelial barrier during intestinal inflammation: Quest for new molecules and mechanisms. Biochimica et Biophysica Acta - Molecular Cell Research, 1864, 1183–1194.

Lee, K.W., Lillehoj, H.S., Jang, S.I., Li, G., Lee, S.H., Lillehoj, E.P., Siragusa, G.R., 2010. Effect of Bacillus-based direct fed microbials on Eimeria maxima infection in broiler chickens. Comp. Immunol. Microbiol. Infect. Dis., 33, pp. e105-e110 20621358.

Lee, J., Yun, H.S., Cho, K.W., Oh, S., Kim, S.H., Chun, T., Kim, B., Whang K.Y., 2011. Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp. immune modulation and longevity. Int J Food Microbiology, 148, 80-86.

Lee, S.H., Lillehoj, H.S., Dalloul, R.A., Park, D.W., Hong, Y.H. & Lin, J.J., 2007. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poult. Sci., 86, 63-66.

Lee, C.C., Wu, C.C., Lin, T.L., 2012. Characterization of chicken melanoma differentiation-associated gene 5 (MDA5) from alternative translation initiation. Comparative Immunology, Microbiology and Infectious Diseases, 35, 335–343.

Lee, B., Moon, K.M., Kim, C.Y., 2018. Tight junction in the intestinal epithelium: Its association with diseases and regulation by phytochemicals. Journal of Immunology Research, 2018, 1–11.

Lee, J. S., Tato, C. M., Joyce, B., Gulen, M. F, Cayatte, C., Chen, Y., Blumenschein, W., Judo, M., Ayanoglu, G., McClanahan, T., Li, X. & Cua, D. J., 2015. Interleukin-23-independent IL-17 production regulates intestinal epithelial permeability. Immunity, 43, 727–738.

Li, Z., Wang, W., Liu, D., Guo, Y., 2018. Effects of Lactobacillus acidophilus on the growth performance and intestinal health of broilers challenged with Clostridium perfringens. Journal of Animal Science and Biotechnology, 9, 1–10.

Lin, R., Heylbroeck, C., Pitha, P.M., Hiscott, J., 1998. Virus-Dependent Phosphorylation of the IRF-3 Transcription Factor Regulates Nuclear Translocation, Transactivation Potential, and Proteasome-Mediated Degradation. Mol Cell Biology, 18: 2986–2996.

Lin, R., Mamane, Y., Hiscott, J., 2000. Multiple regulatory domains control IRF-7 activity in response to virus infection. J. Biol. Chem., 275, 34320-34327.

Lillehoj, H.S., 1998. Role of T lymphocytes and cytokines in coccidiosis. In: International Journal for Parasitology. Int J Parasitol, 1071–1081.

Lillehoj, H.S., Erik, PL., 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Diseases, 44, 408–425.

Lillehoj, H.S., Min, W., Dalloul, R.A., 2004. Recent progress on the cytokine regulation of intestinal immune responses to Eimeria. In: Poultry Science. Poultry Science Association, 611–623.

Liu, D., Guo, Y., Wang, Z., Yuan, J., 2010. Exogenous lysozyme influences *Clostridium perfringens* colonization and intestinal barrier function in broiler chickens. Avi. Pathol, 39, 17–24.

Liu, T., Zhang, L., Joo, D., Sun, S.C., 2017. NF-κB signaling in inflammation. Signal Transduction and Targeted Therapy, 2, 1–9.

Lopez-Osorio, S., Chaparro-Gutierrez. J. J., Gomez-Osorio, L. M., 2020. Overview of Poultry *Eimeria* Life Cycle and Host-Parasite Interactions. Front Vet Sci, 7, 384.

Ludwiczek, O., Vannier, E., Borggraefe, I., Kaser, A., Siegmund, B., Dinarello, C.A., & Tilg, H., 2004. Imbalance between interleukin-1 agonists and antagonists: relationship to severity of Inflammatory bowel disease. Clin. Exp. Immunol., 138, 323-329.

Livak, K. J., & Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25, 402–408.

Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., 2004. TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation. Am J Physiol Gastrointest Liver Physiol, 286, G367–G376.

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A., 2015. Type I interferons in infectious disease. Nat Rev Immunol, 15, 87–103.

Macdonald, S. E., Nolan, M. J., Harman, K., Boulton, K., Hume, D. A., Tomley, F. M., Stabler, R. A., & Blake, D. P., 2017. Effects of *E. tenella* infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology, PLoS ONE*,* 12.

Manabe, A., Furukawa, C., Endo, S., Marunaka, K., Nishiyama, T., Fujii, N., Tabuchi, Y., Matsunaga, T., Ikari, A., 2017. Chlorpheniramine increases paracellular permeability to marker fluorescein lucifer yellow mediated by internalization of occludin in murine colonic epithelial cells. Biological and Pharmaceutical Bulletin, 40, 1299–1305.

Marchiando, A.M., Graham, W.V., Turner, J.R., 2010a. Epithelial barriers in homeostasis and disease. Annual Review of Pathology: Mechanisms of Disease, 5, 119–144.

Marchiando, A.M., Shen, L., Vallen Graham, W., Weber, C.R., Schwarz, B.T., Austin, J.R., Raleigh, D.R., Guan, Y., Watson, A.J.M., Montrose, M.H., Turner, J.R., 2010b. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. Journal of Cell Biology, 189, 111–126.

Markowiak P, Śliżewska K., 2017. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients, 9, E1021.

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E., 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Diary, 16, 189–199.

Martin, M.J., Thottathil, S.E., Newman, T.B., 2015. Antibiotics overuse in animal agriculture: A call to action for health care providers. American Journal of Public Health, 105, 2409–2410.

Marugan-Hernandez, V., Long, E., Blake, D., Crouch, C., Tomley, F., 2017. *Eimeria tenella* protein trafficking: differential regulation of secretion versus surface tethering during the life cycle OPEN. Nature, 7, 1–13.

Majer, O., Liu, B., Barton, G.M., 2017. Nucleic acid-sensing TLRs: trafficking and regulation. Current Opinion in Immunology, 44, 26–33.

Massier, L., Blüher, M., Kovacs, P., Chakaroun, R.M., 2021. Impaired Intestinal Barrier and Tissue Bacteria: Pathomechanisms for Metabolic Diseases. Frontiers in Endocrinology, 12, 1.

Matter, K., Balda, M.S., 2003. Signalling to and from tight junctions. Nature Reviews Molecular Cell Biology, 4, 225–236.

McReynolds, J., C. Waneck, J. Byrd, K. Genovese, S. Duke, D. Nisbet, 2009. Efficacy of multistrain direct-fed microbial and phytogenic products in reducing necrotic enteritis in commercial broilers. Poultry Science, 88, 2075-2080.

Milatz, S., Krug, S. M., Rosenthal, R., Günzel, D., Müller, D., Schulzke, J.-D., Amasheh, S., & Fromm, M., 2010. Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1798.

Mingzhe, G., Liqing, Y., Tao, Y., Lin, H., Qingchao, L., Peilan, L., Tianyu, G., Xia, J., Hui, X., Guangxun, M., Jin, Z,, & Yongfen, X., 2020. IL-1β Enhances the Antiviral Effect of IFN-α on HCV Replication by Negatively Modulating ERK2 Activation. ACS Infectious Dis., 6, 1708-1718.

Munoz, M., Eidenschenk, C., Ota, N., Wong, K., Lohmann, U., Kühl, A.A., Wang, X., Manzanillo, P., Li, Y., Rutz, S., Zheng, Y., Diehl, L., Kayagaki, N., van Lookeren-Campagne, M., Liesenfeld, O., Heimesaat, M., Ouyang, W., 2015. Interleukin-22 Induces Interleukin-18 Expression from Epithelial Cells during Intestinal Infection. Immunity, 42, 321–331.

Neal-McKinney, J.M., Lu X., Duong, T., Larson, C.L., Call, D.R. & Shah, D.H., 2012. Production of organic acids by probiotic *lactobacilli* can be used to reduce pathogen load in poultry. PLoS One. 7, e43928.

Ogawa, A., Andoh, A., Araki, Y., Bamba, T., & Fujiyama, Y., 2004. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. Clinical Immunology, 110, 55–62.

Odenwald, M.A., & Turner, J.R., 2016. The intestinal epithelial barrier: a therapeutic target? Nature Reviews Gastroenterology & Hepatology, 14, 9–21.

Okamoto, R., & Watanabe, M., 2004. Molecular and clinical basis for the regeneration of human gastrointestinal epithelia. Journal of Gastroenterology, 39, 1–6.

Olnood, C.G., Beski, S.S.M., Iji, P.A., Choct, M., 2015. Delivery routes for probiotics: Effects on broiler performance, intestinal morphology and gut microflora. Animal Nutrition, 1, 192–202.

Paris, L., Tonutti, L., Vannini, C., Bazzoni, G., 2008. Structural organization of the tight junctions. Biochimica et Biophysica Acta – Biomembranes, 1778, 646–659.

Park, S.S., Lillehoj, H.S., Allen, P.C., Dong, W.P., FitzCoy, S., Bautista, D.A., Lillehoj, E.P., 2008. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avi. Dis., 52, 14–22.

Park, I., Lee, Y., Goo, D., Zimmerman, N.P., Smith, A.H., Rehberger, T., 2020. The effects of dietary *Bacillus subtilis* supplementation, as an alternative to antibiotics, on growth performance, intestinal immunity, and epithelial barrier integrity in broiler chickens infected with *Eimeria maxima*. Poultry Science, 99, 725–33.

Patel, R.M., Myers, L.S., Kurundkar, A.R., Maheshwari, A., Nusrat, A., Lin, P.W., 2012. Probiotic Bacteria Induce Maturation of Intestinal Claudin 3 Expression and Barrier Function. The American Journal of Pathology, 180, 626.

Pender, C.M., Kim, S., Potter, T.D., Ritzi, M.M., Young, M., Dalloul, R.A., 2017. In ovo supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poultry Science, 96, 1052–1062.

Petecchia, L., Sabatini, F., Usai, C., Caci, E., Varesio, L., & Rossi, G. A., 2012. Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway. Laboratory Investigation, 92, 1140–1148.

Pichlmair, A., Schulz, O., Tan, C., Näslund, T., Liljeström, P., Weber, F., Reis E Sousa, C., 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science, 314: 997–1001.

Pham, H.S.H., Hatabu, T., 2021. Eimeria tenella Infection Modulates the Expression Levels Of Intestinal Epithelial Barrier-Related Genes In Chicken. Journal of Environmental Science for Sustainable Society, 10, 13-16.

Rajput, D.S., Zeng, D., Khalique, A., Rajput, S.S., Wang, H., Zhao, Y., Sun, N., Ni, X., 2020. Pretreatment with probiotics ameliorate gut health and necrotic enteritis in broiler chickens, a substitute to antibiotics. AMB Express, 10, 1–11.

Rao, R., 2009. Occludin phosphorylation in regulation of epithelial tight junctions. Annals of the New York Academy of Sciences, 1165, 62–68.

Rao, R.K., Basuroy, S., Rao, V.U., Karnaky, K.J., Gupta, A., 2002. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-β-catenin complexes from the cytoskeleton by oxidative stress. Biochemical Journal, 368, 471–481.

Rehder, D., Iden, S., Nasdala, I., Wegener, J., Brickwedde, M.K.M.Z., Vestweber, D., Ebnet, K., 2006. Junctional adhesion molecule-A participates in the formation of apico-basal polarity through different domains. Experimental Cell Research, 312, 3389–3403.

Reid, A. J., Blake, D. P., Ansari, H. R., Billington, K., Browne, H. P., Bryant, J., Dunn, M., Hung, S. S., Kawahara, F., Miranda-Saavedra, D., Malas, T. B., Mourier, T., Naghra, H., Nair, M., Otto, T. D., Rawlings, N. D., Rivailler, P., Sanchez-Flores, A., Sanders, M., Pain, A., 2014. Genomic analysis of the causative agents of coccidiosis in domestic chickens. Genome Research, 24, 1676–1685.

Ritzi, M. M., Abdelrahman, W., Van-Heerden, K., Mohnl, M., Barrett, N. W., & Dalloul, R. A., 2016. Combination of probiotics and coccidiosis vaccine enhances protection against an *Eimeria* challenge. Veterinary Research, 47, 1–8.

Rothwell, L., Muir, W., Kaiser, P., 2010. Interferon-γ is expressed in both gut and spleen during *Eimeria tenella* infection, 29, 333–342.

Ruemmele, F.M., Seidman, E.G., Lentze, M.J., 2002. Regulation of intestinal epithelial cell Apoptosis and the pathogenesis of inflammatory bowel disorders. J. of Pediatric Gas. and Nutri, 34, 254–260.

Russel, P.H., Dwivedi, P.N., Davison, T.F., 1997. The effects of cyclosporin A and cyclophosphamide on the populations of B and T cells and virus in the Harderian gland of chickens vaccinated with the Hitchner B1 strain of Newcastle disease virus. Vet Immunol Immunopathology, 60, 171-185.

Sadeghi, A., Toghyani, M., Gheisari, A., 2015. Effect of various fiber types and choice feeding of fiber on performance, gut development, humoral immunity, and fiber preference in broiler chicks. Poultry Science, 94, 2734–2743.

Sato M, Hata N, Asagiri M, Nakaya T, Taniguchi T, Tanaka N., 1998. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS Letters, 441, 106–110.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.R.-D., Fromm, M., Takano, H., Noda, T., Tsukita, S., 2000. Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands. Molecular Biology of the Cell, 11, 4131–4142.

Sawada, N., 2013. Tight junction-related human diseases. Pathology International, 63, 1–12.

Sazawal, S., Hiremath, G., Dhingra, U., Malik, P., Deb, S., Black, R.E., 2006. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infectious Diseases, 6, 374–382.

Scharl, M., Paul, G., Barrett, K.E., McCole, D.F., 2009. AMP-activated protein kinase mediates the interferon-γ-induced decrease in intestinal epithelial barrier function. Journal of Biological Chemistry, 284, 27952–27963.

Schneider, M.R., Dahlhoff, M., Horst, D., Hirschi, B., Trülzsch, K., Müller-Höcker, J., Vogelmann, R., Allgäuer, M., Gerhard, M., Steininger, S., Wolf, E., Kolligs, F.T., 2010. A key role for E-cadherin in intestinal homeostasis and paneth cell maturation. PLoS ONE, 5, 1–13.

Schulzke, J.D., Gitter, A.H., Mankertz, J., Spiegel, S., Seidler, U., Amasheh, S., Saitou, M., Tsukita, S., Fromm, M., 2005. Epithelial transport and barrier function in occludin-deficient mice. Biochimica et Biophysica Acta (BBA) – Biomembranes, 1669.

Segawa, S., Nakakita, Y., Takata, Y., Wakita, Y., Kaneko, T., Kaneda, H., Watari, J., Yasui, H., 2008. Effect of oral administration of heat-killed *Lactobacillus brevis* SBC8803 on total and ovalbumin-specific immunoglobulin E production through the improvement of Th1/Th2 balance. Int J Food Microbiology, 121, 1-10.

Servant, M.J., Tenoever, B., Lin, R., 2002. Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. Journal of Interferon and Cytokine Research, 22, 49–58.

Sharma, S., Azmi, S., Iqbal, A., Nasirudullah, N., Mushtaq, I., 2015. Pathomorphological alterations associated with chicken coccidiosis in Jammu division of India. J of Parasitic Dis., 39, 147-151.

Shen, L., 2012. Tight junctions on the move: Molecular mechanisms for epithelial barrier regulation. Annals of the New York Academy of Sciences, 1258, 9–18.

Shen, L., Weber, C.R., Raleigh, D.R., Yu, D., Turner, J.R., 2011. Tight Junction Pore and Leak Pathways: A Dynamic Duo. Annual Review of Physiology, 73, 283–309.

Shin, K., Fogg, V.C., Margolis, B., 2006. Tight junctions and cell polarity. Annual Review of Cell and Developmental Biology, 22, 207–235.

Shin, K., Margolis, B., 2006. ZOning out Tight Junctions. Cell, 126, 647–649.

Shokryazdan, P., Sieo, C.C., Kalavathy, R., Liang, J.B., Alitheen, N.B., 2014. Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. Biomed Res. Int. Biomed Res Int, 2014, 927268.

Simakachorn, N., Pichaipat, V., Rithipornpaisarn, P., Kongkaew, C., Tongpradit, P., Varavithya, W., 2000. Clinical evaluation of the addition of lyophilized, heat-killed Lactobacillus acidophilus LB to oral rehydration therapy in the treatment of acute diarrhea in children. Journal of Pediatric Gastroenterology and Nutrition, 30, 68–72.

Slifer, Z. M., & Blikslager, A. T., 2020. The integral role of tight junction proteins in the repair of injured intestinal epithelium. International Journal of Molecular Sciences, 21.

Sultan, M., Ben-Ari, Z., Masoud R, Pappo O, Harats D,Kamari Y, and Safran M., 2017. Interleukin-1alpha and Interleukin-1beta play a central role in the pathogenesis of fulminant hepatic failure in mice. PLoS One, 12, e0184084.

Specian, R.D., Oliver, M.G., 1991. Functional biology of intestinal goblet cells. American Journal of Physiology - Cell Physiology, 260.

Stetson DB, Medzhitov R., 2006. Type I Interferons in Host Defense. Immunity, 25, 373–381.

Sunada, Y., Nakamura, S., Kamei, C., 2007. Effects of *Lactobacillus acidophilus* Strain L-55 on Experimental Allergic Rhinitis in BALB/c Mice. Biological & Phar. Bulletin, 30, 2163–2166.

Sunada, Y., Nakamura, S., Kamei, C., 2008. Effect of Lactobacillus acidophilus strain L-55 on the development of atopic dermatitis-like skin lesions in NC/Nga mice. International Immunopharmacology, 8, 1761–1766.

Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cellular and Molecular Life Sciences, 70, 631–659.

Tada, A., Zelaya, H., Clua, P., Salva, S., Alvarez, S., Kitazawa, H., Villena, J., 2016. Immunobiotic Lactobacillus strains reduce small intestinal injury induced by intraepithelial lymphocytes after Toll-like receptor 3 activation. Inflammation Research, 65, 771–783.

Teng, P.Y., Fuller, A.L., Kim, W.K., 2020. Evaluation of nitro compounds as feed additives in diets of Eimeria-challenged broilers in vitro and in vivo. Poultry Science, 99, 1320–1325.

Teng, P. Y., Yadav, S., Castro, F. L. de S., Tompkins, Y. H., Fuller, A. L., & Kim, W. K., 2020. Graded *Eimeria* challenge linearly regulated growth performance, dynamic change of gastrointestinal permeability, apparent ileal digestibility, intestinal morphology, and tight junctions of broiler chickens. Poultry Science, 99, 4203–4216.

Teng, P. Y., Choi, J., Tompkins, Y., Lillehoj, H., Kim, W., 2021. Impacts of increasing challenge with Eimeria maxima on the growth performance and gene expression of biomarkers associated with intestinal integrity and nutrient transporters. Vet Res, 52, 81.

Thompson, M., Kaminski, J., Kurt-Jones, E., Fitzgerald, K., 2011. Pattern recognition receptors and the innate immune response to viral infection. Viruses, 3, 920–40.

Timmerman, H.M., Koning, C.J.M., Mulder, L., Rombouts, F.M., Beynen, A.C., 2004. Monostrain, multistrain and multispecies probiotics - A comparison of functionality and efficacy. International Journal of Food Microbiology, 96, 219–233.

Timmerman, H.M., Veldman, A., van den Elsen, E., Rombouts, F.M., Beynen, A.C., 2006. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poultry Science, 85, 1383–1388.

Tierney J., Gowing H. and Van Sinderen D.V., 2004. *In vitro* inhibition of *Eimeria tenella* invasion by indigenous chicken. *Lactobacillus* species. Vet. Parasitol., 122, 171-182.

Tsukita, Sachiko, Katsuno, T., Yamazaki, Y., Umeda, K., Tamura, A., Tsukita, Shoichiro, 2009. Roles of ZO-1 and ZO-2 in Establishment of the Belt-like Adherens and Tight Junctions with Paracellular Permselective Barrier Function. Annals of the New York Academy of Sciences, 1165.

Utech, M., Ivanov, A.I., Samarin, S.N., Bruewer, M., Turner, J.R., Mrsny, R.J., Parkos, C.A., Nusrat, A., 2005. Mechanism of IFN-γ-induced endocytosis of tight junction proteins: Myosin II-dependent vacuolarization of the apical plasma membrane. Mol. Biology of Cell, 16, 5040–5052.

Upasani, V., Scagnolari, C., Frasca, F., Smith, N., Bondet, V., Vanderlinden, A., Lay, S., Auerswald, H., Heng, S., Laurent, D., Ly, S., Duong, V., Antonelli, G., Dussart, P., Duffy, D., Cantaert, T., 2020. Decreased Type I Interferon Production by Plasmacytoid Dendritic Cells Contributes to Severe Dengue. Front. Immunol., 11, 605087.

Van Itallie, C. M., & Anderson, J. M., 2006. Claudins and epithelial paracellular transport. Annual Review of Physiology, 68, 403–429.

Van Itallie, C. M., Fanning, A. S., Holmes, J., & Anderson, J. M., 2010. Occludin is required for cytokine-induced regulation of tight junction barriers. J of Cell Science, 123, 2844–2852.

Van Baarlen, P., Troost, F., van der Meer, C., Hooiveld, G., Boekschoten, M., Brummer, R.J.M., Kleerebezem, M., 2011. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proceedings of the National Academy of Sciences of the United States of America, 108, 4562–4569.

Vantsawa, P.A., Umar, T., Bulus, T., 2017. Efefcts of Probiotic Lactobacillus Acidophilus on Performance of Broiler Chickens. Direct Research Journal of Agriculture and Food Science 5.

Vetrano, S., Danese, S., 2009. The role of JAM-A in inflammatory bowel disease: Unrevealing the ties that bind. Annals of the New York Academy of Sciences, 1165, 308–313.

Vicas, L., 2010. Antibiotic Sensitivity Of The Escherichia Coli Strains Isolated From Infected Skin Wounds, 58, 637–645.

Virman, J., Soini, Y., Kujala, P., Luukkaala, T., Salminen, T., Sunela, K., Kellokumpu-Lehtinen, P.L, 2014. Claudins as prognostic factors for renal cell cancer. Anticancer Res., 34, 4181–4187.

Wada, M., Tamura, A., Takahashi, N., & Tsukita, S., 2013. Loss of claudins 2 and 15 from mice causes defects in paracellular Na+ flow and nutrient transport in gut and leads to death from malnutrition. Gastroenterology, 144, 369–380.

Walter, J., 2008. Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. Applied and Environmental Microbiology, 74, 4985–4996.

Wang, F., Graham, W.V., Wang, Y., Witkowski, E.D., Schwarz, B.T., Turner, J.R., 2005. Interferon-γ and tumor necrosis factor-α synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. American Journal of Pathology, 166, 409–419.

Wang, L., Li, L., Lv, Y., Chen, Q., Feng, J., Zhao, X., 2018. *Lactobacillus plantarum* Restores Intestinal Permeability Disrupted by Salmonella Infection in Newly-hatched Chicks. Scientific Reports, 8.

Wang, Yaya, Mumm, J.B., Herbst, R., Kolbeck, R., Wang, Yue, 2017. IL-22 Increases Permeability of Intestinal Epithelial Tight Junctions by Enhancing Claudin-2 Expression. The Journal of Immunology, 199, 3316–3325.

Williams, J.M., Duckworth, C.A., Burkitt, M.D., Watson, A.J.M., Campbell, B.J., Pritchard, D.M., 2015. Epithelial Cell Shedding and Barrier Function: A Matter of Life and Death at the Small Intestinal Villus Tip. Veterinary Pathology, 52, 445–455.

Wiryawan, K.G., Marianeni, S., Sriasih, M., 2014. Probiotics or Mixed Herbs as Alternativesto Antibiotics for Meat Chicken II, 581–584.

Willis WL, Isikhuemhen OS, Ibrahim SA., 2007. Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotics. Poultry Science, 86, 1856–1860.

Withanage, G. S. K., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell, D., & McConnell, I., 2004. Rapid Expression of Chemokines and Proinflammatory Cytokines in Newly Hatched Chickens Infected with *Salmonella enterica* Serovar Typhimurium. Infection and Immunity, 72, 2152–2159.

Willemsen, L. E. M., Hoetjes, J. P., Van Deventer, S. J. H., & Van Tol, E. A. F., 2005. Abrogation of IFN-γ mediated epithelial barrier disruption by serine protease inhibition. Clinical Experimental Immunology, 142, 275–284.

Wolk, K., Witte, E., Wallace, E., Döcke, W.D., Kunz, S., Asadullah, K., Volk, H.D., Sterry, W., & Sabat, R., 2006. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. European Journal of Immunology, 36.

Wu, Y., Wang, B., Zeng, Z., Liu, R., Tang, L., Gong, L., Li, W., 2019. Effects of probiotics *Lactobacillus plantarum 16* and *Paenibacillus polymyxa 10* on intestinal barrier function, antioxidative capacity, apoptosis, immune response, and biochemical parameters in broilers. Poult Sci, 98, 5028–5039.

Yadav, S. Bajagai, Athol V. Klieve, Peter J. Dart, Wayne L. Bryden, 2016. Probiotics in animal nutrition: production, impact and regulation. FAO, Rome.

Yan, Z., Ming-Xue, Z., Zhi-Yong, X., Huan-Cheng, X., Xiao-Zhen, C., Sha-Sha, Y., Wen-Long, Z., Shan, L., Qiang-Hua, L., Rui, B., 2015. Relationship between Eimeria tenella development and host cell apoptosis in chickens. Poultry Science, 94, 2970–2979.

Ye, D., Ma, I., Ma, T.Y., 2006. Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier. American Journal of Physiology - Gastrointestinal and Liver Physiology, 290.

Ye, D., & Ma, T. Y., 2008. Cellular and molecular mechanisms that mediate basal and tumor necrosis factor-α induced regulation of myosin light chain kinase gene activity. J Cell Mol Med, 12, 1331–1346.

You, M., 2014. The comparative analysis of infection pattern and oocyst output in Eimeria tenella, E. maxima and E. acervulina in young broiler chicken. Veterinary World, 7, 542–547.

Yu, B., Chang, P., Lee, T., 2014. Feeding Probiotic and Herb to Late Pregnant Sow to Affect Piglet Growth Performance , Fecal Score and Diarrhea Incidence on Suckling Pig II, 2063–2066.

Yu, D., Marchiando, A.M., Weber, C.R., Raleigh, D.R., Wang, Y., Shen, L., Turner, J.R., 2010. MLCK-dependent exchange and actin binding region-dependent anchoring of ZO-1 regulate tight junction barrier function. Proceedings of the National Academy of Sciences of the USA, 107, 8237–8241.

Yu, Q., Wang, Z., Yang, Q., 2012. Lactobacillus amylophilus D14 protects tight junction from enteropathogenic bacteria damage in Caco-2 cells. Journal of Dairy Science, 95, 5580–5587.

Yun, C.H., Lillehoj, H.S., Choi, K.D., 2000a. *Eimeria tenella* infection induces local gamma interferon production and intestinal lymphocyte subpopulation changes. Infection and Immu., 68, 1282–1288.

Yun, C.H., Lillehoj, H.S., Lillehoj, E.P., 2000b. Intestinal immune responses to coccidiosis. Developmental and Comparative Immunology, 24, 303–324.

Zhou, B., Jia, L., Wei, S., Ding, H., Yang, J., Wang, H., 2020. Effects of *Eimeria tenella* infection on the barrier damage and microbiota diversity of chicken cecum, Poultry Science, 99, 1297-1305.

Zeuthen, L.H., Christensen, H.R., Frøkiær, H., 2006. Lactic acid bacteria inducing a weak interleukin-12 and tumor necrosis factor alpha response in human dendritic cells inhibit strongly stimulating lactic acid bacteria but act synergistically with gram-negative bacteria. Clinical and Vaccine Immunology, 13, 365–375.

Zhang, 1995. In vivo role of tumor necrosis-like factor in *E. tenella* infection. Avi. Dis., 39, 859–866.

Zhang, W., Azevedo, M.S., Wen, K., Gonzalez, A., Saif, L.J., Li, G., Yousef, A.E., Yuan, L., 2008. Probiotic *Lactobacillus acidophilus* enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs. Vaccine, 26, 3655–3661.

Zhen, W., Shao, Y., Gong, X., Wu, Y., Geng, Y., Wang, Z., Guo, Y., 2018. Effect of dietary Bacillus coagulans supplementation on growth performance and immune responses of broiler chickens challenged by Salmonella enteritidis. Poultry Science, 97, 2654–2666.

Zheng, Y., Valdez, P.A., Danilenko, D.M., Hu, Y., Sa, S.M., Gong, Q., Abbas, A.R., Modrusan, Z., Ghilardi, N., de Sauvage, F.J., Ouyang, W., 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nature Medicine, 14, 282–289.

Zhou, B., Wang, H., Wang, X., Zhang, L., Zhang, K., Xue, F., 2010. Eimeria tenella: Effects of diclazuril treatment on microneme genes expression in second-generation merozoites and pathological changes of caeca in parasitized chickens. Experimental Parasitology, 125, 264–270.

Zulkifli, I., Abdulkah, N., Azrin, N.M. & HO, Y.W., 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Br. Poultry Science, 41, 593-597.