Title: The relationship between major symptoms in *Eimeria tenella* early infection time and the disruption of intestinal barrier function via altering gene expression of epithelial junctional molecules in the cecum

HIGHLIGHTS

- Diarrhea by *E. tenella* infection causes by junctional barrier's disruption.
- Higher expression of claudin-2 related with diarrhea in *E. tenella* infected chicks.
- Several junctional molecules relate with bleeding by *E. tenella* infection.

Title: Relationship between *Eimeria tenella* associated-early clinical signs and molecular changes in the intestinal barrier function.

Running title: *Eimeria tenella*'s infection effects on epithelial junctional molecules' gene expression.

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Abstract

The major clinical signs of coccidiosis in chickens due to *Eimeria* parasite are diarrhea and bloody feces. Previous studies showed that the impairment of the intestinal epithelial barrier and the elevation of the intestinal permeability are causes of clinical signs associated with coccidia challenges. Nevertheless, the information about molecular changes of the epithelial barrier at the early stage of the infection with a specific *Eimeria* species has not been mentioned. Hence, this study aims to elucidate the temporal relationships between epithelial barrier conditions and clinical signs in chickens infected with *Eimeria tenella* over the time from the earliest stages of infection.

White Leghorn chickens were inoculated with 1×10^4 oocysts of *E. tenella*. Thereafter the chickens were monitored for their daily clinical signs through observation, and between 5 dpi to 10 dpi, feces were collected for oocysts counting. Chickens were then administrated with fluorescein isothiocyanate-dextran (FITC-d) for gastrointestinal permeability test and tissues were collected each day for histopathological observation and total RNA extraction. Finally, the mRNA expression levels of the tight and adherens junction genes and cytokine genes were evaluated using the quantitative real-time polymerase chain reaction (qRT-PCR).

In this study, clinical signs such as diarrhea and bloody feces were observed concurrently from 3 to 8 dpi. Histopathology changes such as severe inflammation, hemorrhage, and epithelial desquamation were identified in the cecum specimens. The FITC-d level in the *E. tenella*-infected group was significantly higher than in the control group. In the infected group, the expression of claudin-2 gene was also upregulated, whereas the expressions of claudin-3 and E-cadherin genes were decreased as compared to the control group. These results implied that

clinical signs of avian coccidiosis were associated with the intestinal barrier disruption via changes in expression levels of claudins and E-cadherin at the intestine.

Keywords: adherens junction; bloody feces; diarrhea; *Eimeria tenella;* epithelial barrier; tight junction.

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 and 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, 2017). Adherens junction molecules are involved in strong adhesive bonds between the epithelial cells and intercellular communications (Chida et al., 2009). Therefore, 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.

Materials and Methods

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.

Animals, tissue collection, and experimental design

Eggs (White Leghorn) were purchased from Kui potory 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.

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).

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 1; Erben et al., 2014).

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.

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.

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. 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).

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 $P < 0.01$ (**).

Results

Clinical signs 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 (Figure 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 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).

Histological observations

Infiltrated cells were observed through the mucosa and submucosa from the 3rd to the 6th dpi (Figure 2; arrows). Infiltrated cells also extended through the mucosa and submucosa as well as marked hyperplasia of epithelial cells on the 6th dpi (Figure 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 (Figures 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 3). We observed the epithelial monolayer to be well conserved up to the 5th dpi (Figures 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 (Figure 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.

The 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 (Figures 3A and B). 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 ($P < 0.05$; Figure 3C). 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 3D).

The mRNA expression in *E. tenella*-infected cecum

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 (Figures 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 (Figures 4 and 5).

The OCDN expression levels were significantly reduced on the 4th and 5th dpi compared with the uninfected control group (Figure 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.

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 (Figure 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.

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 (McKenzie et al., 1985), 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 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., 2006; 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 4 indicated that CLDN-1 mRNA level was first decreased at 4th dpi ($P < 0.05$), but later remarkably increased at 5th and 6th dpi ($P < 0.05$). Teng et al. (2020) have reported that CLDN-1 gene expression increases at 6th 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 the acute mechanisms of repair in the injured intestinal epithelium (Slifer & Blikslager, 2020).

The expression of CLDN-2 was significantly higher at 3rd, 4th, and 5th dpi (Figure 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 indicated 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 (Figure 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 (Figures 5 and 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., 2006). 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 (Figure 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.

In 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.

Declaration of Competing Interest

The authors declare no competing financial conflicts of interest.

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Table caption

Table 1. The scoring system for evaluating inflammation level.

Table 2. The sequence of primer pairs used for amplification of target genes. Note: RPS17 (Ribosomal protein S17); JAM-2 (Junctional adhesion molecule 2); ZO-1 (Zonula occludins 1); E-cadherin (Epithelial cadherin); IL (Interleukin); TNF-α (Tumor necrosis factor-α); IFN-γ (Interferon γ).

Table 3. Feces observation and histomorphology lesion score evaluation.

Figure legends

Figure 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).

Figure 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) Three dpi. Magnitude is × 200; (B) Four dpi. Magnitude is × 200; (C) Five dpi. Magnitude is × 200; (D) Six dpi. Magnitude is × 200.

Figure 3. The concentration of FITC-d in the 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. $^{a, b, c}$ different superscripts show the significance between the days at $P < 0.05$.

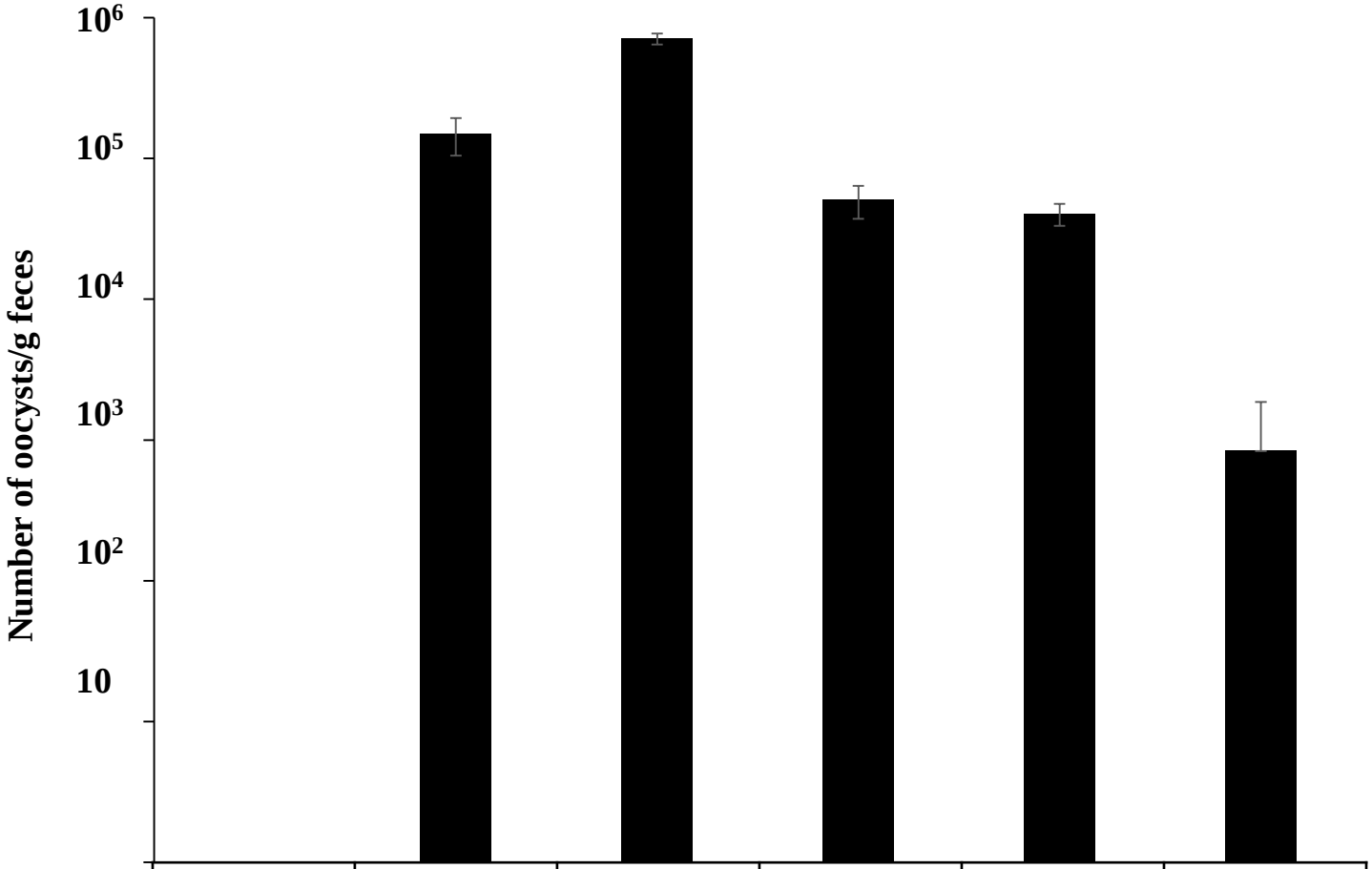
Figure 4. The mRNA expression levels of claudins in ceca of chicken with *E. tenella*. Control group, chicks in this group were not treated as a control; *E. tenella*-infected group, chicks in this group were orally infected with 1×10^4 oocysts of *E. tenella*. The open column represents the control 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. (*: $P < 0.05$; **: $P < 0.01$).

Figure 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 6. The mRNA expression levels of Cytokine 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. (*: $P < 0.05$).

Figure

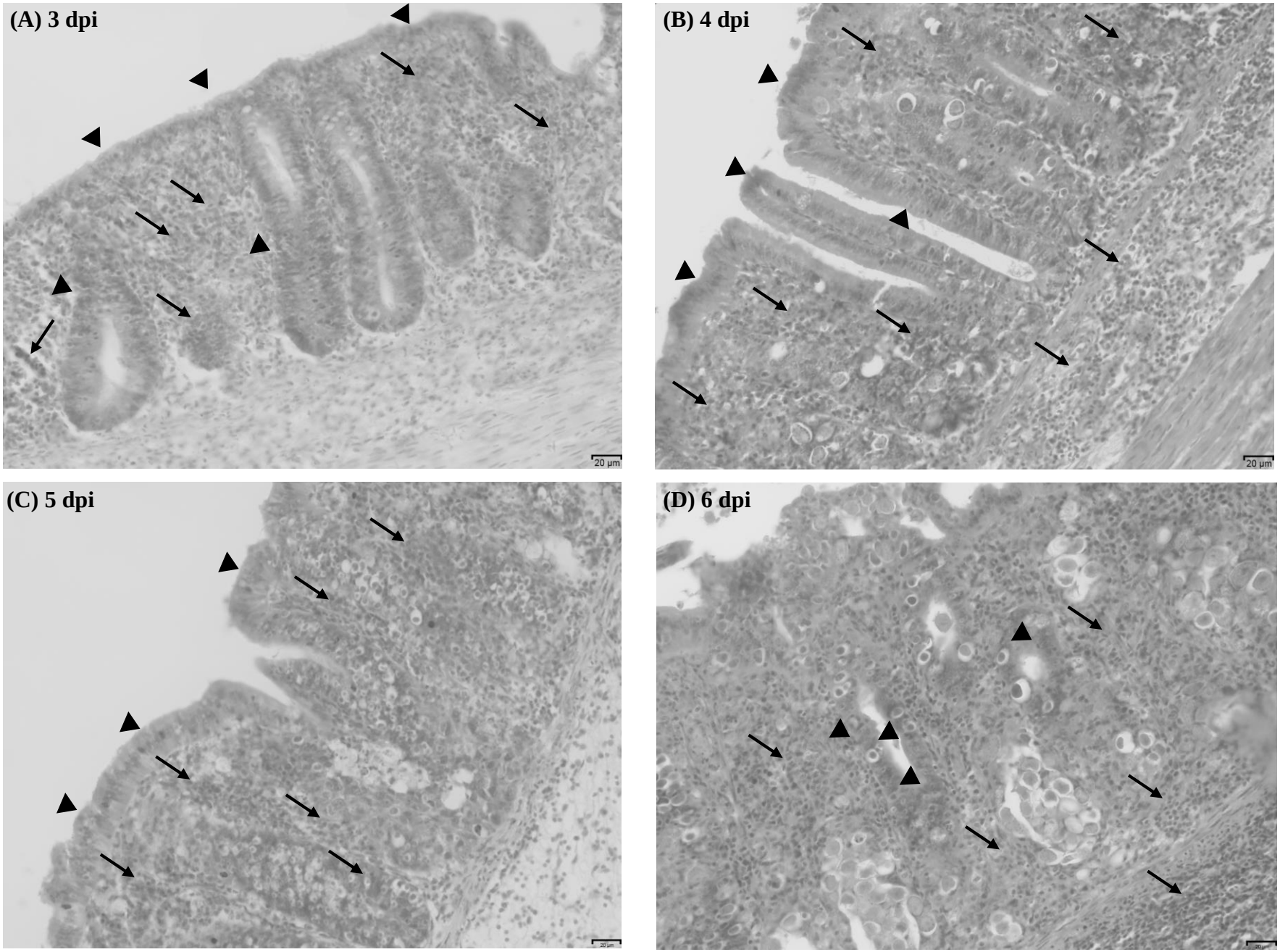
Hung HSP et al., Fig. 1



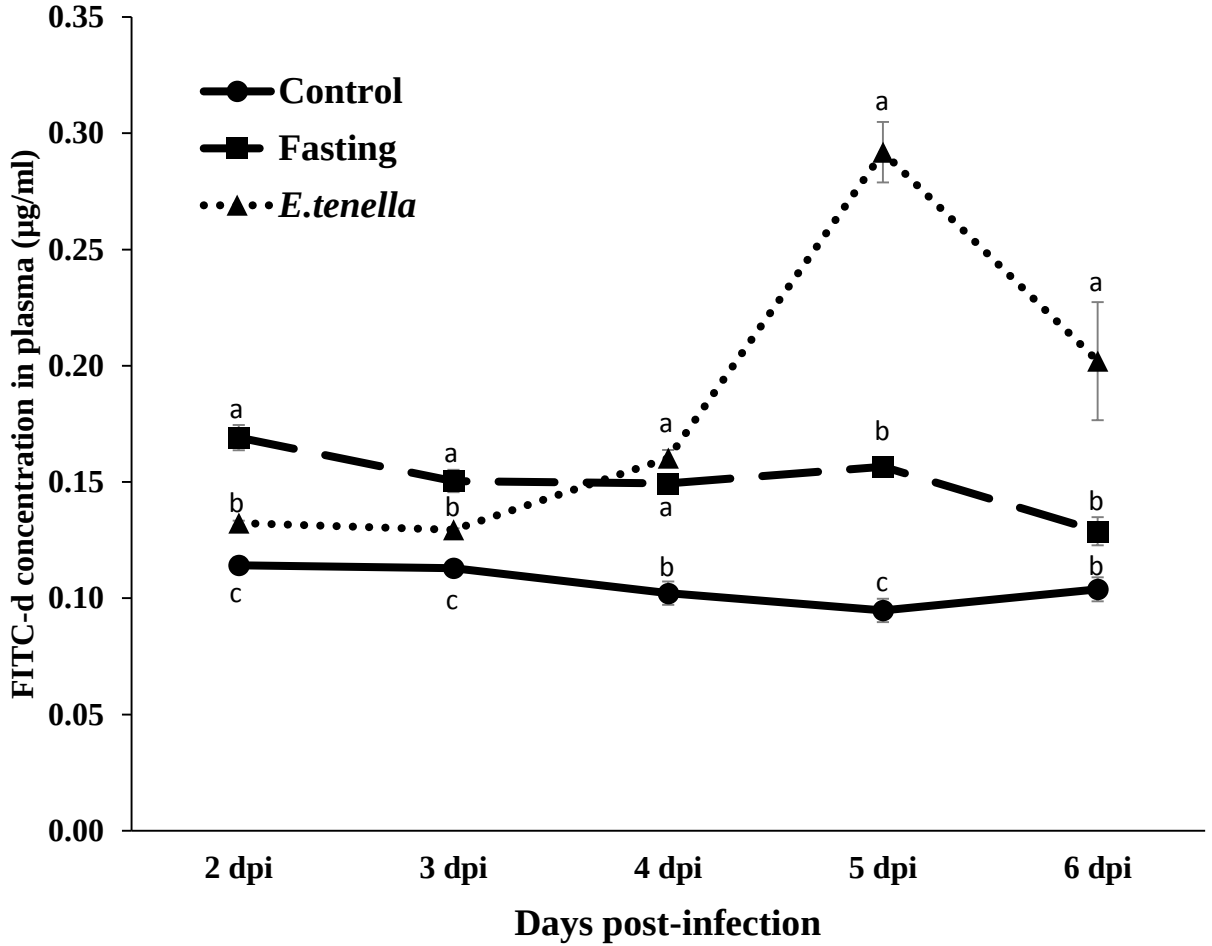
5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
ND	149250 ± 18726	708750 ± 43225	50583 ± 5452	40333 ± 2929	833 ± 422

ND: Not detected

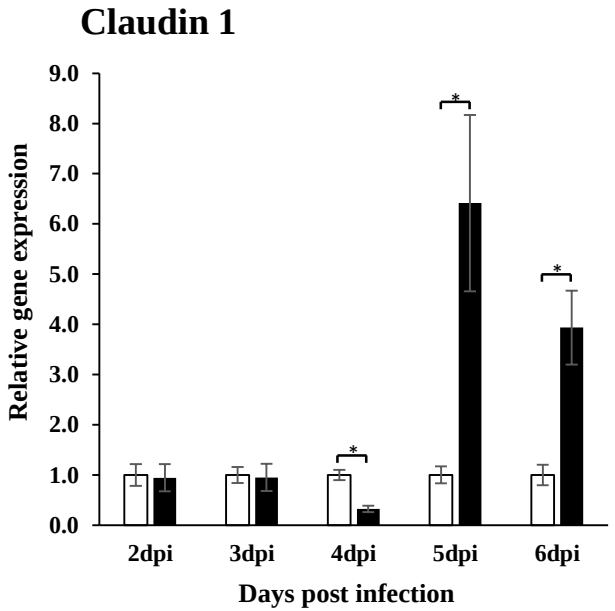
Hung HSP et al., Fig. 2

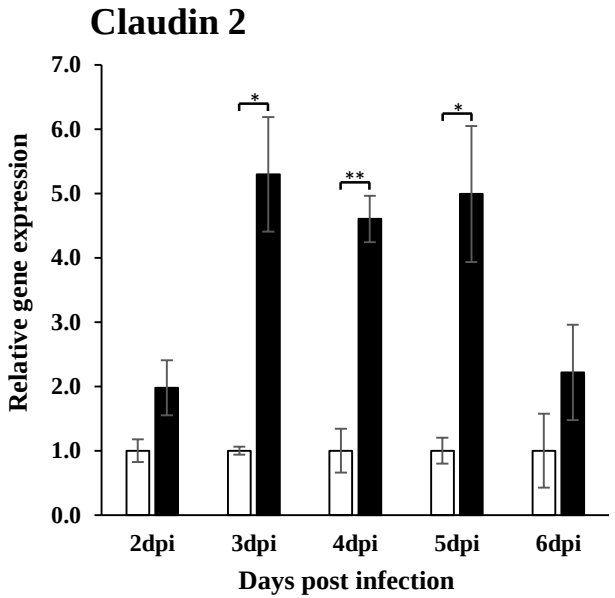


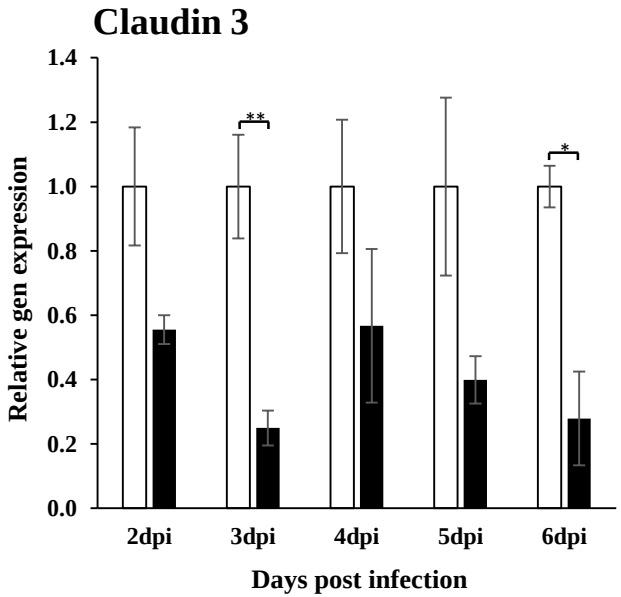
Hung HSP et al., Fig. 3

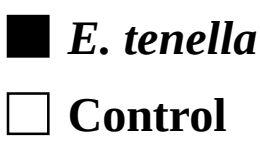


Hung HSP et al., Fig. 4

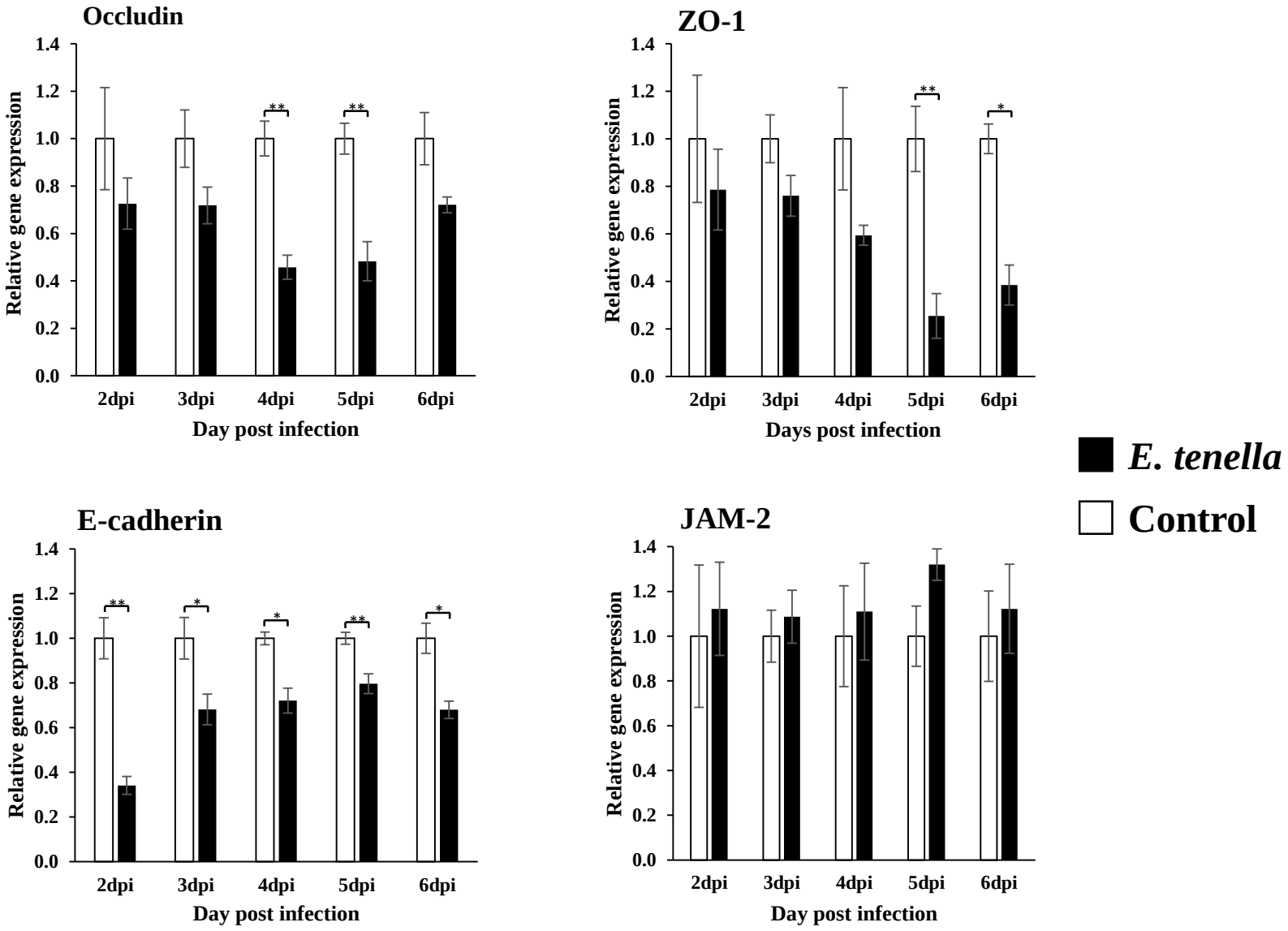




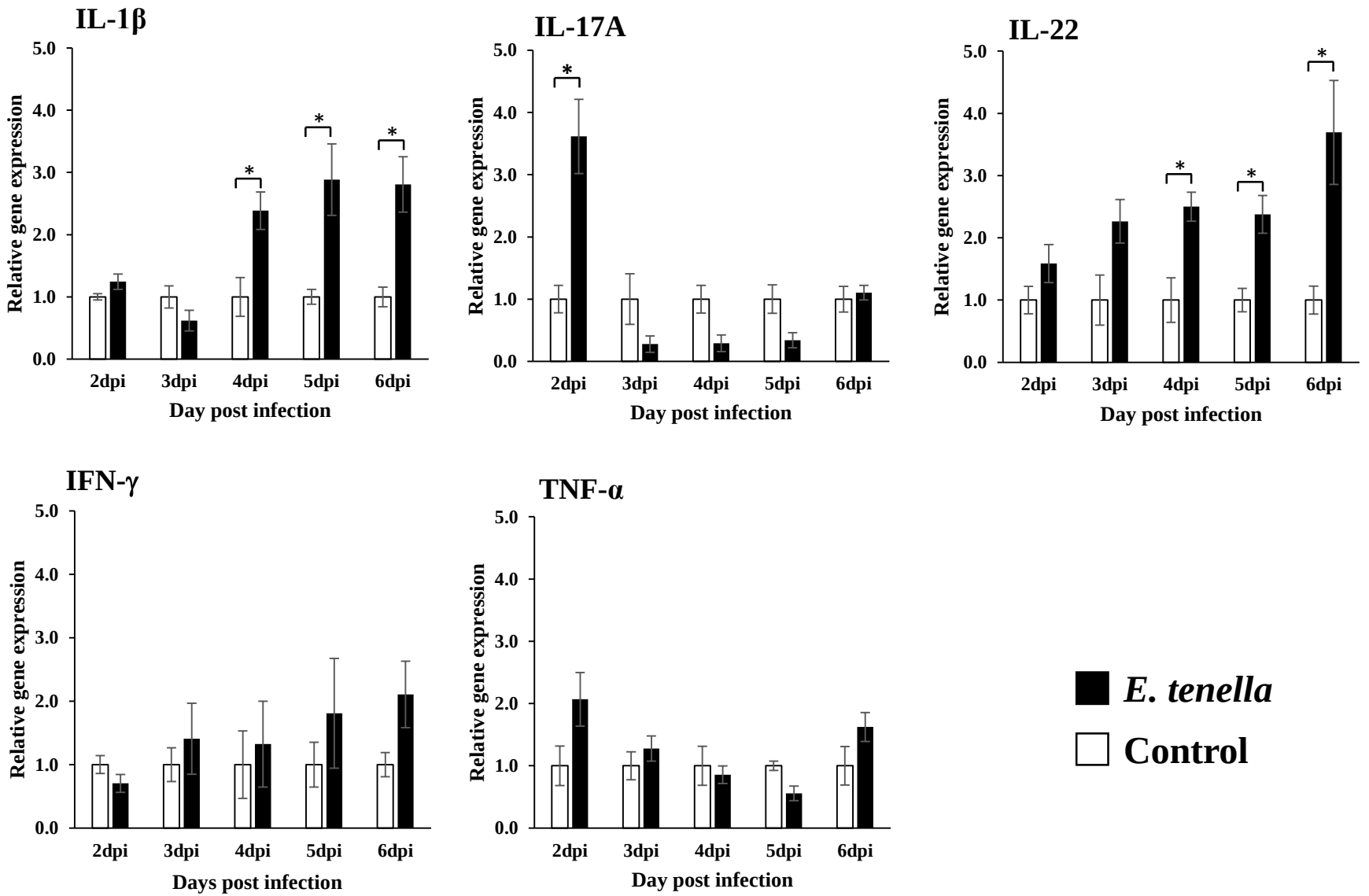




Hung HSP et al., Fig. 5



Hung HSP et al., Fig. 6



Table

Table 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

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

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

Table 3. Feces observation and histomorphology lesion score evaluation.

Day post infection		1	2	3	4	5	6	7	8	9	10
Symptoms	**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