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Intestinal barrier and mucosal immunity in broilers, Thai Betong, and native Thai Praduhangdum chickens

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Abstract: The intestinal barrier includes physical and chemical components for preventing the invasion of pathogenic and toxic agents. The aim of this study was to investigate small intestinal morphology, antimicrobial peptides, and tight junction (TJ) distribution among broilers, Thai Betong chickens, and native Thai Praduhangdum chickens. Intestinal samples from 40 chickens of each breed were collected. The results of the histological and morphological examination revealed that the duodenum of all breeds had the maximum villus height compared to the other parts. The intestinal tract of both Betong and Praduhangdum chickens was low in the number of mucin and goblet cells. In broilers, the intestinal surface mucins correlated with the number of β-defensin-positive Paneth cells. Claudin-1 protein was observed in the cytoplasm of the epithelium in all breeds, with the highest intensity of claudin-1 staining detected in the intestinal cells of the ileum. Localization of occludin was higher in the broilers in all intestinal segments than in the native Thai chickens. In conclusion, the broilers exhibited a large amount of TJ proteins and mucosal immunity as compared to native Thai-breed chickens. TJ localizations together with intestinal morphology and mucosal immunity can be utilized as biological markers for gut health.

Key words: Chicken, intestinal barrier, intestinal morphology, mucosal immunity, tight junction

1. Introduction

The intestinal epithelium acts as a protective barrier against the invasion of intraluminal microorganisms and toxins. Gut health is defined according to the physical and chemical components constituting the intestinal barrier, as well as by its ability to selectively absorb nutrients into the body (1). The intestinal barrier consists of a mucous layer formed from the secretions of goblet cells, intestinal epithelial cells (IECs), and secretory molecules such as antimicrobial peptides produced by Paneth cells present at the base of the crypt of Lieberkühn (1,2). The intestinal tract is covered by a columnar layer of epithelial cells, which are connected by tight junctions (TJs) and the junctional complex. TJs play an important role as interlinks between intercellular spaces (3,4). Various proteins in TJs act as selective channels that regulate the paracellular transport of water, ions, and small molecules. Claudins, occludin, zonula occludin proteins, and desmosomes regulate epithelial permeability and contribute to the maintenance of the barrier integrity of the intestinal tracts of animals and humans (4,5). The intestinal cell lining is covered by a mucous layer that prevents the colonization of intestinal microbes on the epithelial surface. Mucus and mucins, secreted from goblet cells, together with secretions from Paneth cells containing antimicrobial peptides and lysozyme, function in innate immune response (1). The loss of TJs and the junctional complex may affect intestinal permeability by decreasing the protective barrier. This can be used as an indicator for alterations in the intestinal epithelium (6). In chickens, an impaired intestinal barrier can lead to poor growth performance due to inefficient nutrient digestion and absorption (7). Several studies have noted that mRNA expression and the levels of serum proteins, e.g., claudin-1, occludin, ZO-1, and cytokines, can be used in broilers as biomarkers to determine the function of the gut barrier (7,8).

The consumption of Thai chicken meat has been increasing every year due to its toughness, high collagen mass, and low cholesterol and fat compared with the meat texture of broilers (9,10). These unique features and the high demand could lead to an increase in the mass production of Thai chicken meat in the commercial sector in the future. The major challenge for poultry farms is to improve feed efficiency as this accounts for

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about 60%–70% of total production costs. As mentioned above, fundamental information regarding intestinal morphology and particularly the gut health of Thai-breed chickens needs to be evaluated. This is also essential as native Thai chickens are genetically different from broilers. Such studies can therefore provide useful information to improve the growth performance of Thai-breed chickens in the future. Therefore, the objective of this study was to investigate the histological morphology, mucous layer, and localization of TJ proteins in the intestinal epithelium among three chicken breeds, including commercial broilers (Arbor Acres), Thai Betong, and native Thai Praduhangdum chickens. This study will lead to a better understanding of the morphology of the intestinal barrier along the proximal and distal parts of the small intestine.

2. Materials and methods

2.1. Animals and experimental design

A total of 120 chickens (120 days old, 40 per breed) of three breeds, including commercial broilers (Arbor Acres), Thai Betong (KU line), and native Thai Praduhangdum chickens, were procured from the Kasetsart University poultry farm in Bangkok, Thailand. The birds were raised in cages with four replications per group, each containing ten birds. Light and climatic conditions were observed according to the recommendations of the Animal Ethics Committee of Kasetsart University, Thailand. Feed and water were provided ad libitum with a starter diet from days 1 to 14, a grower diet from days 15 to 21, and a finisher diet from day 21 until sacrifice. The ingredients and nutrient contents of the diets are listed in Table 1. All birds were fasted for 12 h before the collection of samples, after which seven birds per group were randomly selected and euthanized. Intestinal contents were removed and rinsed with 0.01 M phosphate-buffered saline (PBS) at a pH of 7.4. Tissue samples were collected from the duodenum (the midpoint of the duodenal loop), jejunum (3 cm proximal and distal from Meckel's diverticulum), and ileum (2 cm proximal from the ileocecal fold). All samples were fixed with 10% neutral-buffered formalin in PBS for 24 h and dissected into sections of 1 cm^2.

2.2. Histology

The paraffin sections were cut at thicknesses of 3–5 μm and stained with hematoxylin and eosin for histological examination. Intestinal morphology, villus height, crypt depth, and villus height to crypt depth ratio were evaluated. Periodic acid–Schiff (PAS) staining was used to detect mucin glycoproteins, calculate the density of goblet cells as the number of goblet cells per unit of surface area (mm^2), and measure the intensity of the mucin intestinal surface. Twenty well-oriented villi and crypts were determined per cross-section of each intestinal segment. The investigators were double-blinded for evaluation of all morphologic and histological observations.

2.3. Immunohistochemistry

Paraffin-embedded sections mounted on positively charged slides were deparaffinized, and antigens were retrieved by incubating the slides in citrate buffer at a pH of 6.0 at 95 °C. Endogenous peroxidase activity was quenched with 3% H_2O_2 in distilled water, followed by blocking of the nonspecific background using 2% bovine serum albumin at room temperature (RT). Subsequently, the slides were incubated separately overnight at 4 °C in a humidified chamber with the following primary antibodies: rabbit anticlaudin-1 (1:100, Cell Marque, USA), rabbit-anti-β-defensin (1:200, Santa Cruz Biotechnology, USA), and

Table 1. Ingredients and nutrient content of the basal diet (dry mass basis).

Ingredient	Starter (%)	Grower (%)	Finisher (%)
Rice	46.6	-	-
Corn	-	41.8	46
Bran meal	10	10	14
Soybean meal	30.7	-	-
Fish meal	8	6	6
Extruded full fat soybean	-	39.2	31.1
Dicalcium phosphate	0.5	1.2	1.2
Limestone meal	0.6	1	1
Oil	2.8	-	-
DL-methionine	0.2	0.2	0.1
Salt	0.35	0.35	0.35
Mineral and vitamin premix	0.25	0.25	0.25
Total	100	100	100
Energy (kcal/kg)	3150	3150	3150

rabbit-antioccludin (1:100, Santa Cruz Biotechnology). The slides were then incubated with a horseradish peroxidase-conjugated goat antirabbit antibody (EnVision, Dako, Denmark) at RT for 1 h, followed by development with diaminobenzidine (DAB) chromogen (Invitrogen, USA). The sections were counterstained with hematoxylin. PBS (0.01 M) was used as a negative control. The localization of TJ and β-defensin proteins was determined by randomly selecting five fields per section using an Olympus FSX100 microscope (Olympus, Japan). The positively stained cells and areas were detected using the digital image analysis Cell-D program (Olympus) and calculated as an intensity score (11).

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significant differences in the histological parameters of villus height, crypt depth, villus height/crypt depth ratio, goblet cells density, and β-defensin-positive Paneth cells in the three breeds. This was followed by a comparison using Tukey's test. The Kruskal–Wallis test with Dunn's multiple comparisons post test was used to determine the significant difference of the intensity of mucin surface and the intensity score of claudin-1 and occludin localizations. All statistical analyses were performed using GraphPad Prism, Version 5.0 (GraphPad Software, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Intestinal morphology

The intestinal morphometric variables of villus height, crypt depth, villus height/crypt depth ratio, and goblet cell density are presented in Table 2. The villus height of the duodenum in the three breeds was found to be the maximum as compared to other intestinal segments ($P < 0.05$). The villus height of the ileum of the native Thai Praduhangdum chickens was the smallest compared to the others ($P < 0.05$). The villus height/crypt depth ratio, goblet cell density, and mucin surface accumulation were significantly higher in all of the intestinal segments of the broilers compared to the native Thai Praduhangdum and Betong chickens ($P < 0.05$). The PAS-positive areas were

Table 2. The intestinal morphology of broiler, Thai Betong, and native Thai Praduhangdum chickens.

Parameters	Intestinal segment	Broiler	Thai Betong	Native Thai Praduhangdum
Villus height (μm)	Duodenum	1,221.00 ± 89.12^{b}	968.30 ± 71.59^{a}	1,081.00 ± 43.94^{a}
	Jejunum	935.70 ± 64.30	835.30 ± 33.26	797.50 ± 58.90
	Ileum	892.20 ± 43.94^{e}	762.30 ± 44.85^{e}	646.90 ± 36.61^{f}
Crypt depth (μm)	Duodenum	241.80 ± 15.87^{a}	265.10 ± 18.75^{a}	681.00 ± 43.94^{b}
	Jejunum	147.20 ±9.84^{d}	229.30 ±32.38^{d}	597.50 ± 58.90^{d}
	Ileum	177.40 ± 28.54^{e}	191.70 ± 16.30^{e}	346.90 ± 76.61^{f}
Villus height/crypt depth ratio	Duodenum	4.91 ± 0.38^{b}	3.67 ± 0.26^{a}	3.74 ± 0.26^{a}
	Jejunum	5.96 ± 0.75^{d}	3.95 ± 0.39^{c}	3.87 ± 0.22^{c}
	Ileum	4.20 ± 0.50^{f}	3.48 ± 0.36^{e}	3.08 ± 0.26^{e}
Goblet cells density (mm^2)	Duodenum	667.80 ± 23.45^{b}	462.50 ± 34.35^{a}	541.50 ± 42.87^{a}
	Jejunum	810.00 ± 14.95^{d}	510.00 ± 42.68^{c}	515.70 ± 24.05^{c}
	Ileum	735.30 ± 42.99^{f}	670.80 ± 30.48^{e}	637.80 ± 25.05^{e}
Intensity of mucin intestinal surface	Duodenum	33.74 ± 7.50^{b}	16.55 ± 4.52^{a}	12.47 ± 5.38^{a}
	Jejunum	28.69 ± 4.30^{d}	14.60 ± 5.56^{c}	11.86 ± 3.45^{c}
	Ileum	36.28 ± 6.90^{f}	11.65 ± 3.87^{e}	10.80 ± 4.65^{e}
β-defensin-positive Paneth cells (mm^2)	Duodenum	157.45 ± 13.04^{b}	112.89 ± 7.56^{a}	98.54 ± 5.30^{a}
	Jejunum	175.07 ± 30.50^{c}	141.85 ± 9.40^{c}	107.67 ± 10.83^{d}
	Ileum	180.77 ± 12.58^{f}	137.67 ± 27.32^{e}	103.49 ± 17.45^{e}

Values are presented as mean ± SEM.

^{a,b} Parameter values of duodenum and ^{b} indicates a significant difference between the three breeds ($P < 0.05$).

^{c,d} Parameter values of jejunum and ^{d} indicates a significant difference between the three breeds ($P < 0.05$).

^{e,f} Parameter values of ileum and ^{f} indicates a significant difference between the three breeds ($P < 0.05$).

detected at the apical sites of intestinal cells and within the goblet cells, as shown in Figures 1A–1I. However, a correlation between the number of goblet cells and the mucous surface was not observed.

3.2. β-Defensin, claudin-1, and occludin distributions of intestinal mucosa

The number of β-defensin-positive cells and the intensity score of claudin-1 and occludin are presented in Table 2 and in Figures 1 and 2. β-Defensin was expressed in the cytoplasm of Paneth cells in the crypt of Lieberkühn (Figures 1G–1I). However, localization did not extend to the villi of all segments. A large number of β-defensin-positive Paneth cells were observed in the jejunum and ileum of the broilers. Claudin-1 localization was observed in the cytoplasm at the apical to basolateral sites of the intestinal cells (Figures 2A–2C). The distribution of sparse granules

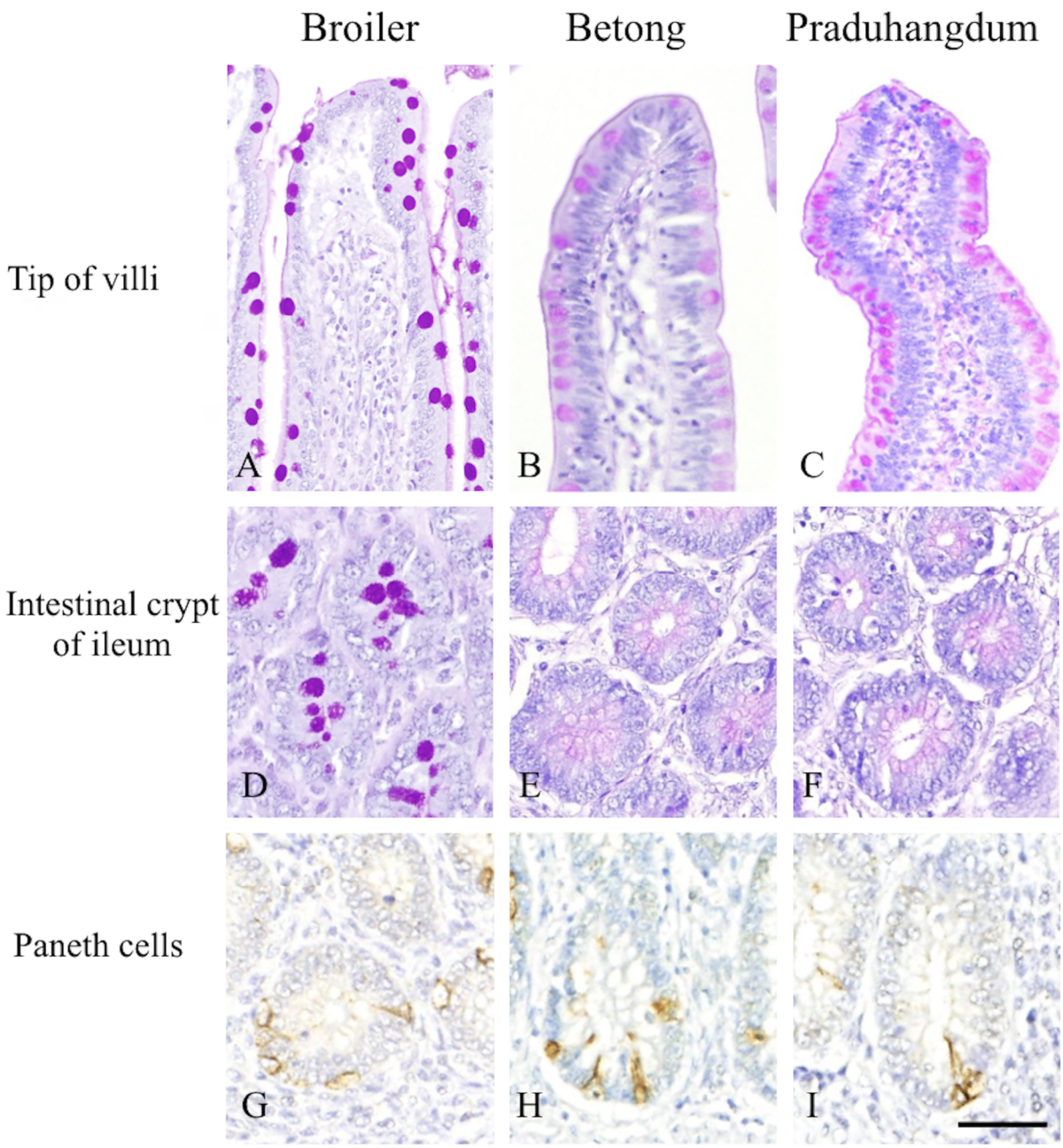

Figure 1. The morphology of PAS-positive goblet cells and the mucin layer at the tip of villi and crypts of ileum and β-defensin in Paneth cells of broiler (A, D, G), Thai Betong (B, E, H), and native Thai Praduhangdum (C, F, I) chickens, respectively. The bar indicates a width of 30 μm.

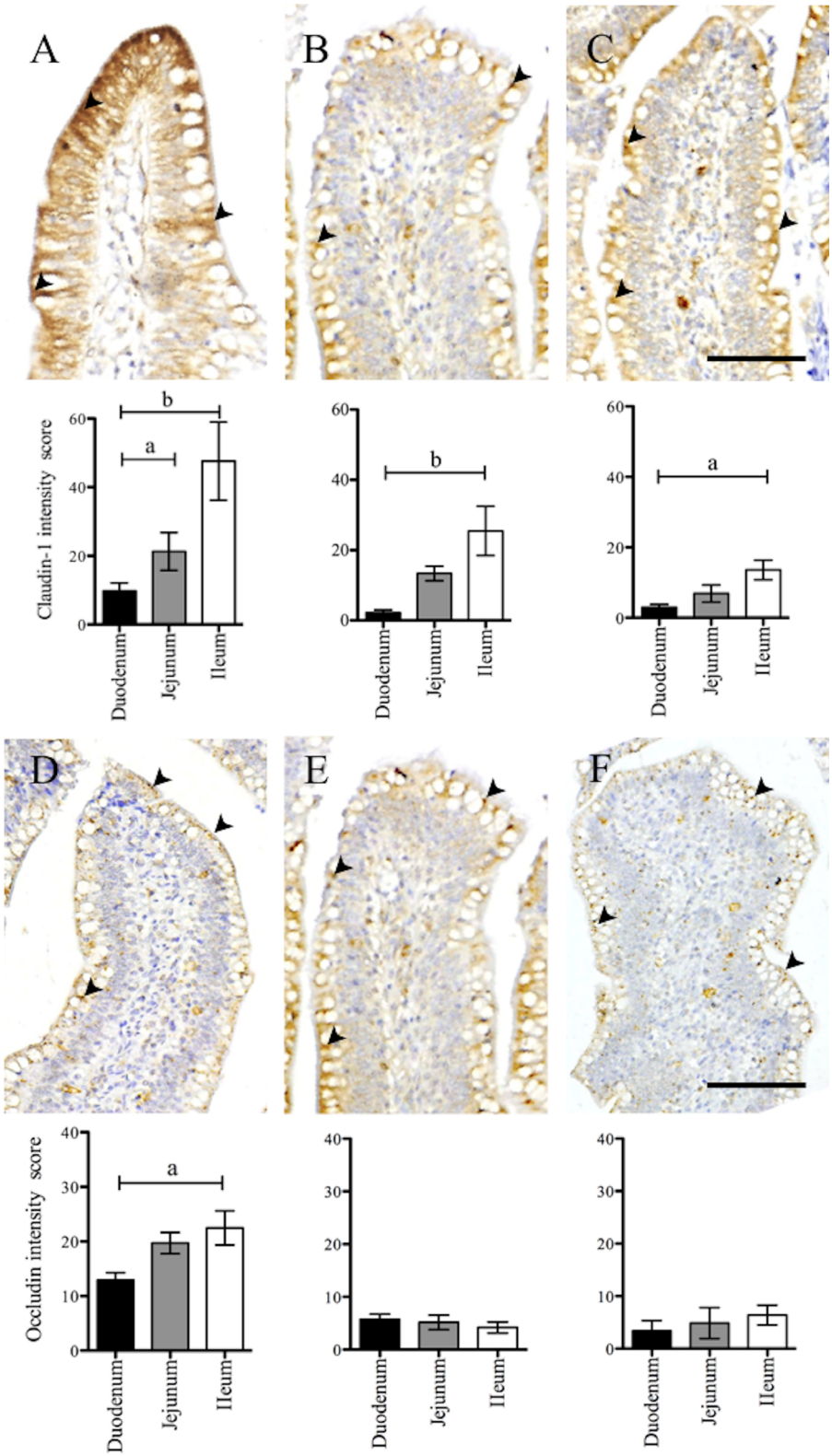

Figure 2. Claudin-1 and occludin expression in the ileum of broiler (A, D), Thai Betong (KU line) (B, E), and native Thai Praduhangdum (C, F) chickens. Claudin-1 and occludin are diffused with brown granules from the basolateral site to the apical site of the cytoplasm (arrow heads). The TJ intensity scores of each intestinal segment are presented below (^{a} = $P < 0.05$, ^{b} = $P < 0.01$). The bars indicate a width of 30 μm.

was seen in the cytoplasm of the epithelium, whereas these granules could not be detected in the goblet cells. The staining was more pronounced in the distal jejunum and ileum. It was significantly higher in the ileum of all breeds compared to other segments ($P < 0.05$). The intensity of claudin-1 localization in the intestinal tract of the native Thai Praduhangdum and Betong chickens was significantly lower than in the broilers ($P < 0.05$). Occludin was also found to be present in all of the intestinal segments of the broilers in a granular pattern. The expression was significantly higher than that found in the Thai chickens ($P < 0.05$), as shown in Figures 2D–2F. The localization of TJ proteins and antimicrobial peptides in the native Thai Praduhangdum and Betong chickens was lower compared to commercial broilers. These results indicated the presence of poor intestinal barrier function and gut immunity in the native Thai Praduhangdum and Betong chickens.

4. Discussion

Intestinal morphology, the mucous layer, and TJ proteins were studied in three healthy chicken breeds raised under normal conditions to reduce the effects of diet ingredients and microbial challenges. This study showed that the mucin protective surface was significantly higher in the commercial broilers and especially in the ileum compared to both Thai-breed chickens. Our results correspond to a previous study reporting that the mucin intestinal surface was not different among intestinal segments (12). However, the fasting period affected the adherent mucin on the intestinal surface and resulted in high degradation of the mucus layer and villus structure (12,13). Therefore, it is possible that Thai-breed chickens might be affected by fasting (12 h) before sample collection, which caused a reduction in the mucin intestinal surface and intestinal morphology. Different types of diets can affect the intraluminal milieu by disturbing the digestive viscosity and mucins (7). However, all chickens in our study were fed the same diet and nutrients to reduce the effect of dietary factors.

The β-defensin protein is only found in avian species. It is produced in secretory granules by Paneth cells, which secrete lysozyme and phospholipase A2 (14). It is also found in the intestinal crypts of chickens (15). Antimicrobial defensin peptides from Paneth cells form a gradient within the mucosal layer on the epithelial surface (14) and aid antimicrobials against both gram-negative and positive bacteria (8,15–18). The temporary weakening of the mucosal surface and lower amount of β-defensin resulted in the intestine being more penetrable to intraluminal bacteria.

The paracellular spaces of IECs are sealed with intestinal TJs. Claudin and occludin proteins are mainly responsible for intestinal barrier function and paracellular diffusion (1,7,8,19). Claudin-1 proteins are constantly expressed in the intestinal lining from the hatching period to the formation of adult chickens (20). It has been documented that the use of feed additives effectively enhanced the integrity of the intestinal barrier. Improved mucosal immune responses were also observed in broilers when challenged with pathogenic bacteria (8,17,21). Impairment of TJ proteins in broilers has been found to be associated with intestinal disruption, but studies on the role of claudin-1 and occludin in the small intestine are still limited (8). The current study aimed to determine the morphologic localization of TJ and mucosal proteins and describe their effect on the gut health of different strains of chickens from Thailand. Expression levels of claudin-1 and occludin proteins in two Thai-breed chickens were significantly lower than in commercial broilers. Impaired TJs also contribute to diarrhea by causing intestinal leakage via the flux mechanism (22). In addition, a previous study reported that an impaired TJ barrier is consistently associated with growth performance (23). This demonstrates that low TJ protein levels allow the entry of intraluminal antigens into the systemic circulation, resulting in poor intestinal permeability. Poor performance, especially poor growth efficacy, has been observed in native Thai Praduhangdum and Thai Betong (KU line) breeds (9,10,24). Therefore, intestinal TJ proteins could improve gut integrity and nutrient absorption, thereby increasing the growth rate in Thai-breed chickens. TJs are also related to innate mucosal immunity, protecting the host from bacterial colonization and invasion to the submucosa (8,17). It was found that these molecules were present in significantly low numbers in Thai-breed chickens.

This study found that TJ localization was apparently abundant in the distal small intestine, but less so in the duodenum. IECs are dynamic and undergo constant epithelial shedding affected by gastric juice. Crypt stem cells are subsequently generated from intestinal cells to the villi tips to maintain barrier function (1). TJs are also redistributed to the cell membrane and fill the paracellular gaps. However, these gaps could not always be filled (25) and appeared as areas with less TJ intensity in the duodenum of the three breeds in our study

In conclusion, this is the first study that reveals the fundamentals of intestinal characteristics and barrier function of two Thai-breed chickens compared to commercial broilers (Arbor Acres). As the native Thai

Praduhangdum and Betong chickens originated in rural areas of Thailand, a lack of genetic selection may affect performance in terms of raising the animals for mass production. This study showed that TJ proteins and mucosal immune response could be used as biological markers, together with suitable dietary ingredients, to help improve the performance of indigenous chickens.

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