Gut mucosal functions and health in poultry

Workshop of 17th AAAP Animal Science Congress

Date: 24 August, 2016 (9:00-11:00)

Venue: Kyushu Sangyo University (Fukuoka, Japan)

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The gut health is important to maintain good production ability and to obtain safe meat and eggs in poultry industry. The primary role of intestinal mucosal tissue is digestion and absorption of nutrient. Determination of the mechanism by which nutrients and are absorbed and localization of specific small molecules including the collagen-related molecules in the digestive tract is essential to develop the feeding system with the best growth performance. A new approach has shown that the MALDI-TOF MSI is a powerful tool to evaluate the localization of nutrition in terms of anatomical and physiological research. In addition, the intestinal mucosa carries out immune functions against pathogenic bacteria that may be present in the ingesta. Innate and adaptive immune systems are involved in forming the intestinal mucosal barrier. The proper composition and form of feed is essential for maximum performance. Since antibiotics use as a feed additive was proven to be unsafe and has been banned in many countries, the need to an effective alternatives is important. Recently, reports suggest that probiotics use can reduce the intestinal pathogenic microbes. If probiotics and microbiomes enhance the mucosal immune functions, they could be the excellent candidates for safe feed additives to strengthen the mucosal barrier functions. In this session we will exchange the latest knowledge on the gut mucosal functions, and discuss the benefit of pre- and probiotics for poultry intestinal health.

Speakers and titles

New parameters and evaluation: Collagen-related molecules in chicken intestine.
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New parameters and evaluation: Collagen-related molecules in chicken intestine

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Introduction

Morphological analysis is simple and pragmatic methods, which gives us meaning of phenomena on absorption of nutrition and defense from the pathogen and virus in digestive organ. However, these anatomical results are not satisfied intrinsic interest, and it is not enough to evaluate nutrient absorption in chicken intestine using scanning electron microscope (SEM) and transmission electron microscope. Most of the nutrient are small molecules, and digested by the different process both mechanical and chemical breakdown. Intestinal small molecules are effective and functional. However, protective functions for the pathogen and virus may require flexible block key function on the surface.

Recently, macro molecules have been possible to separate using infrared absorbance peaks and FT-IR spectroscopic imaging methods, applied to evaluate collagen maturation, mineralization and calcification on the bone (osseous) tissue and cartilage (Boskey *et.al.* 2007). Generally, animal bone cell has widely separated and surrounded by large amounts of the matrix which contains hydroxyapatite, calcium carbonate and collagen fibers. The feature of FT-IR imaging is that collagen fibril organization can be evaluated without general and immunohistochemical staining meathods. Interestingly, carcinogenesis tissue has shown the difference IR spectrum compared with normal mouse colon tissue (Cohenford *et.al.* 2012).

This workshop reviews highlight, introduce our feeding experiment results using FT-IR imaging. Previous our feeding experiments indicated both activated nutrient absorption and extracellular matrix (ECM) content. Advanced in our feeding experiment discussed Wood Charcoal Vinegar (WCV, Miyazaki Midori Pharms. inc, Japan) increases ECM expression both chicken muscle and eggshell membrane (Yamauchi *et.al.* 2013, 2014). In this experiment, broiler fed with WCV was used and evaluated the changes of Intestinal epithelial cells (IECs) membrane used by FT-IRI, SEM and light microscope. Accordingly, FTIR imaging results indicated that high absorbance areas of 2500 cm^{-1}, this absorbance expressed O-H bond in carboxylic acid (Boskey *et.al.* 2007), were located on the surface of IECs and boundary between IECs and lamina propria in an experimental group. O-H bonds in carboxylic acids had strong hydrogen bond ability. Carboxyl group (COOH-) was known to be affected ECM adsorption ability by changing surface potential (Lin JH *et.al.* 2014). It may demand innovative approaches to discover ECM-related phenomena on nutritional absorbance mechanism using anatomical methods, and requiring intestinal health in chicken.

The Matrix Assisted Laser Desorption Ionization-Time of Flight/Mass spectroscopy (MALDI-TOF/MS) can identify amino acid sequences of multiple proteins, and this imaging methods is possible to identify the localization and affected proteins based on mass in tissue. Recently, mouse brain researchers established separation using MALDI-TOF/MS imaging methods, localization of glutamic acid and nucleotide derivatives (ATP, ADP and AMP)(Miura *et.al.* 2010). Usually, non-fixed cryo-sections are required for MALDI-TOF/MS, although paraffin sections with trypsin or EDTA treatment are also useful (Ronci *et.al.* 2010). In this

session, introduce our established method of using intestinal paraffin section for MALDI-TOF/MS imaging. Paraffin embedded samples were easy to use for morphological analysis. MALDI-TOF MSI was powerful tool to evaluate the localization of nutrition in terms of anatomical and physiological research.

To evaluate nutrient absorption, FT-IRI is very useful to identify the interaction both ECM and IEC on the surface of an intestine. Therefore, both MALDI-TOF MS imaging and FT-IR imaging merged results may provide a new focus of variable molecules of nutritional absorbance.

FTIR imaging methods and results

Five μm paraffin sections of duodenum are put on BaF_2 glasses. After deparaffinized by xylene for 10 min, one section on BaF_2 glasses (PIKE technologies, the U.S.) were set on FT-IR imaging microscope (Varian 670-IR and 620-IR, Agilent, Japan). FT-IRI of the duodenum villi tops are acquired in transmission mode. In this study, 16 scans are added with wavenumber ranging from 4000 to 900 cm^{-1}. Resolution of FT-IR analysis is 4cm^{-1}. The surface of IECs and boundary between IECs and lamina propria in experimental groups has much absorbance of O-H bands in carboxylic acids in Figure l.

MALDI-TOF MS imaging methods and results

Ten μm paraffin sections of jejunum are put on Indium Tin Oxide (ITO) coated glasses (Bruker Daltonics, Germany) and deparaffinized at 60 degree for 10 minutes. After deparaffinized, dried in desiccator for 1 day and acquired 4800 dpi optical images of sample slide glass used by flatbed scanner (ES-10000G, EPSON). 30 mg/ml 2,5-Dihydroxybenzoic acid (DHB, Bruker Daltonics, Germany) in MeOH / 0.2%TFA (1:1, v/v) is used for matrix solution and sprayed on jejunum sections by Image prep (Bruker). Jejunum sections were inserted in Ultraflextreme (Bruker Daltonics, Germany). In this experiment, MALDI-TOF MSI data is acquired in linear positive mode with 100 um spatial resolutions. The number of laser shots are 250, laser power is 70% and signals from 0 to 2000 m/z are measured. After acquiring the data, reconstituted MALDI-TOF MSI of jejunum by flex imaging ver2.2 (Bruker Daltonics, Germany). Mass of collagen related amino acids (alanine, glycine and prorine) are focused on and investigated the localization of amino acids on jejunum sections. Collagen related amino acids (alanine, glycine and proline) are located on jejunum villi, crypts and smooth muscles in Figure 2.

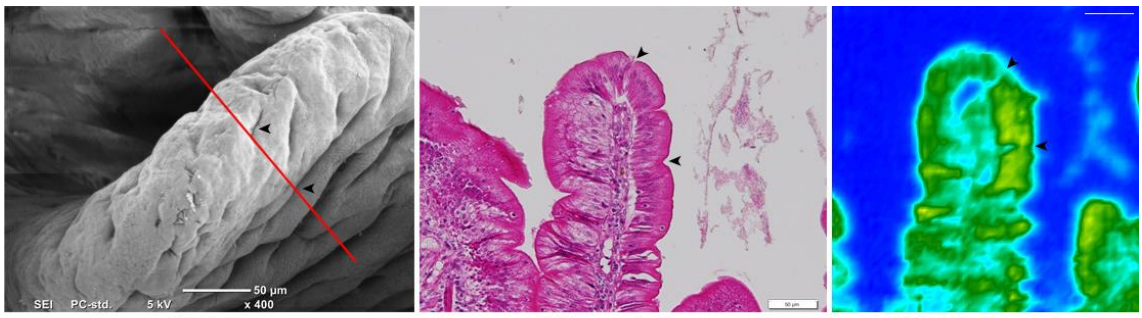

Figure1. Comparison of SEM, HE staining and FT-IR imaging of duodenal villi. Red line means observing sections of HE and FT-IRI. Black arrows showed intussusceptions. Right panel showed 2500 cm^{-1} absorbance imaging of duodenum villi tops. 2500 cm^{-1} indicated O-H bonds of carboxylic acids. Scale bar was 50μm.

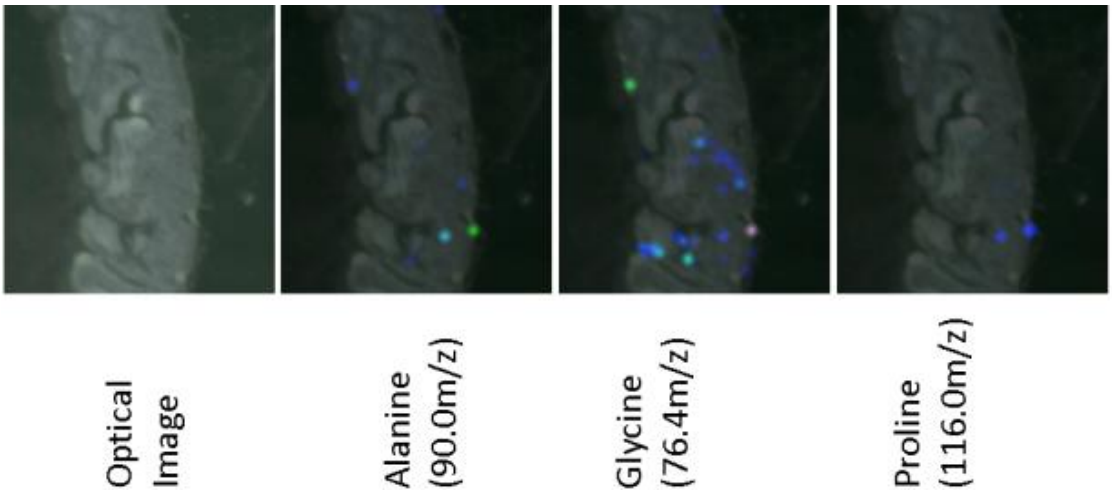

Figure 2. Localization of collagen related amino acids on jejunum sections in control chicken. This figure showed the localization of alanine, glycine and proline on jejunum sections. Color dots indicated localization and intensity (white color was strongest intensity and blue was weak intensity).

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Effect of Supplementing Some Acidifiers in Drinking Water on Gastrointestinal Tract of Poultry and Piglets

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Introduction

Animals reared in a tropical zone are very susceptible to many diseases such as respiratory diseases and gastrointestinal (GI) diseases. Currently, the livestock industry is focusing greater attention towards addressing public concern for environmental and food safety (Gunal et al., 2006), with the potential mutation of antibiotic resistant strains of bacteria being strongly avoided, consequently many alternative feed additives, including enzymes, organic/inorganic acids, probiotics, prebiotics, herbs and etheric oils and immunostimulants are being used.

The effective function of GI tract is influenced by physiological and ecological conditions such as morphology of villi, pH, enzyme secretion, health status, available nutrients as well as the population of various microorganisms (Sarra et al., 1985). In terms of pH in the GI tract, optimal pH in each segment improves gut health and function. For example, optimal pH for pathogenic bacterial growth is close to 7 or slightly higher, while beneficial microorganisms live in an acidic pH (5.8-6.2) and they compete with the pathogens (Ferd, 1974); thus lowering pH by supplemental organic acids improves nutrient absorption (Boling et al., 2001). Organic acid is one of an alternative to antibiotic growth promoters, which is used in the diet to prevent animals from harmful bacteria and to improve production performance. Therefore, organic acids have a beneficial effect on antimicrobial function, digestibility and nutrient resorption (De Freitas *et al*., 2006). However, there are many factors affecting the levels of pH in the GI tract such as age, pH of diet or drinking water, feedstuff, formula of diets and protein or fat level in diets.

Several pathogenic bacteria including *Campyrobacter* spp., *E.* coli, *Salmonella* spp. and *Clostridium* spp. can multiply in dirty water, and cause a great number of diseases and retard growth performance. Reducing pH in drinking water via organic acids supplementation may be an alternative tool for purification of the drinking water for animals. Inducing acidity in drinking water to improve production performance is not a new concept, however the reports of reducing pH of GI tract by organic acids supplementation in drinking water with similar pattern of feedstuff or materials and methodology of pH determination are limited. In this paper, 5 experiments that investigated effects of supplemental organic acids in drinking water on pH of GI tract are summarized and descripted.

Gastrointestinal pH of Some Animal Species

Since composition of diets strongly influence to buffering capacity in GI tract. In order to eliminate this effect, corn-soybean based diets was fed to each species (chicken, duck and piglet). For determination of pH, same materials and methodology were also used. The pH of GI tract of poultry and piglet fed corn-soybean diets are summarized in Figure 1, and optimal pH for pathogenic bacteria growth are shown in Table 1. It is clear that range of pH levels from middle part of small intestine (jejunum) to large intestine (colon or rectum) (pH 6.07-6.30) are still suite for pathogenic growth, and can harmful to digestive and absorptive capacity of nutrients.

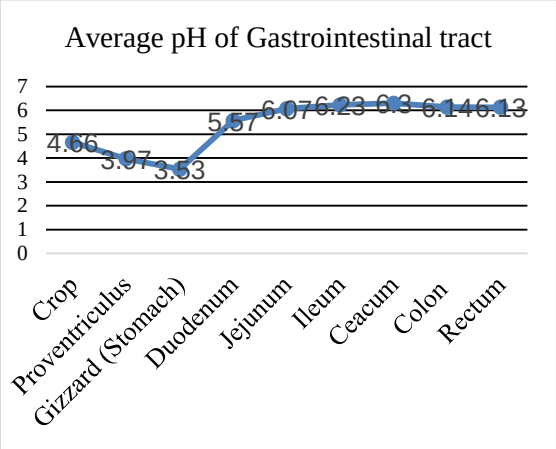

Figure 1. Average pH of GI tract of poultry (chicken and duck) and piglets fed with corn-soybean diets. This figure shows that pH of jejunum, ileum, ceacum, colon or rectum (pig) are appropriated for the development of pathogenic bacteria such as *E. coli*, *Salmonella* spp. or *Clostridium* spp. (see table 1).

Table 1. The Optimal pH for Growth of Some bacteria

Bacteria	Optimal pH
E. coli	6.0-8.0
Salmonella spp.	6.0-7.5
Steptococcus spp..	6.0-7.5
Staphylococcus spp.	6.8-7.5
Clostridium spp.	6.0-7.5
Lactobacillus spp.	5.4-6.4

Source: Dhawale (2005); Hai Meng (2006)

Effect of Acidity in Drinking Water on GI Tract and Growth Performance

The effect of acidity in drinking water on the pH of the GI tract, pathogenic contaminations in drinking water and productive performance are presented in Table 2. Supplementing organic acids did not clearly reduce pH in each segment of the GI tract. Moreover, it seems that the pH of GI tract was reduced only in foregut (from crop to jejunum), while it is conversely increased in hindgut (from ileum to colon) when drinking water with low pH was given. In accordance with Risley et al. (1992) who gave an organic acid to postweaning pigs and did not see a change in gut pH, this may be caused by retention time and contents of the stomach (Walsh et al., 2004), and physiological homeostasis in the tract. Similar with poultry, Hernandez et al. (2006) reported no effect on intestinal pH with the use of organic acids as a result of the strong buffering action of the poultry GI tract. It is suggested that increasing acidity of drinking water by organic supplementation dose not significantly decrease pH throughout the GI tract, and pathogen just slightly declined in hindgut (Data not shows), although supplementing organic acids clearly inhibit the pathogen in drinking water.

Although inducing acidity in drinking water by organic acids dose not directly influence to pH of GI tract, volume of drinking water was depressed in broiler chickens when pH was dropped to 3.33. Formic acid supplementation depressed water consumption and growth rate rather than the MHA-FA. In contrast, piglet consume more water and feed when the pH was decreased at the same level of the chicken, consequent body weight was improved. It is indicated that as long as water intake is not extremely depressed by the acidity, supplementing organic acids in drinking water seems to have benefit on purification of drinking water and productive performance of laying hen and piglets.

Table 2. Effect of Acidity in Drinking Water on Gastrointestinal pH and Performance of poultry and piglet

	Broiler Chicken (Formic acid)		Broiler Chicken (MHA-FA)		Laying Hen (MHA-FA)		Meat-type Duck (MHA-FA)		Piglet (MHA-FA)		Mean (Untreated)	Mean (Treated)	Change
	0%	0.1%	0%	0.1%	0%	0.1%	0%	0.1%	0%	0.1%			
Drinking water pH	7.5	3.5*	7.5	3.2*	7.5	3.2*	7.7	3.3*	6.8	3.3*	7.4	3.3	-4.1
Bacteria in Drinking Water													
E. coli (logCFU/ml)	5.15	0.00*							2.05	0.30*			
Total bacteria count (logCFU/ml)							7.77	3.33*	2.08	1.06*			
Blood and Each Segment of Gastrointestinal Tract pH													
Blood	-	-	-	-	-	-	7.63	7.62	-	-	-	-	-
Proventiculus (Stomach)	4.60	4.64	3.93	3.84	4.72	4.72	3.61	3.11	4.6	4.2	4.29	4.10	-0.19
Gizzard	3.80	4.11	3.21	2.53	4.47	4.07	2.93	2.70	-	-	3.60	3.35	-0.25
Duodenum	3.86	3.77	2.78	2.13	4.39	3.95	6.05	5.82	5.7	5.5	4.55	4.23	-0.32
Jejunum	5.67	5.39	4.74	4.69	5.93	6.04	6.08	5.79*	6.75	6.35	5.83	5.65	-0.18
Ileum	5.58	5.76	4.89	4.81	5.92	6.01	6.67	6.64	6.8	6.8	5.97	6.00	+0.03
Caecum	5.58	6.38	5.33	5.09	6.35	6.29	5.97	6.09	5.98	5.88	5.84	5.94	+0.10
Colon	6.68	6.49	6.40	5.52	6.09	6.30	6.13	5.90	6.10	6.05	6.28	6.05	+0.22
Rectum	-	-	-	-	-	-	-	-	6.13	6.00	-	-	
Mean	**5.11**	**5.22**	**4.61**	**4.26**	**5.41**	**5.34**	**5.34**	**5.15**	**6.00**	**5.82**			
Growth Performance													
Body weight kg or % Egg	2.63	2.43*	2.93	2.97	79.76	82.40	2.91	2.90	31.8	35.9*			
FCR	1.80	1.84	1.71	1.70	2.18	2.10*	2.07	2.09	2.70	2.16			
Water intake (l/d)	0.845	0.742 *	0.525	0.498 *	0.218	0.217	0.831	0.807	2.50	3.49 *			

MHA-FA= DL-Methionine Hydroxy Analogue Free Acid

Table 3 shows villous height and crypt depth in the segment of duodenum. In case of positive response of growth performance cause by an acidity in drinking water, villous growth was promoted and ratio of villous height : crypt depth was also increased.

Table 3. Effect of Acidity in Drinking Water on Duodenal Morphology

	Broiler Chicken (Formic acid)		Broiler Chicken (MHA-FA)		Piglet (MHA-FA)	
	0%	0.1%	0%	0.1%	0%	0.1%
Villous height (μm)	1590.91	1534.29	1511.3	1670.75*	365	433*
Crypt depth (μm)	273.01	223.97	235.02	222.53	464	488
Villous height/ Crypt depth	5.82	6.88	6.43	7.50[#]	0.78	0.88

[#]P=0.06

Conclusion

In conclusion, reducing pH in drinking water by organic acids supplementation clearly reduce pathogenic contaminations in the water, while pH throughout GI tract were not significantly changed. Perhaps the supplementing organic acids in drinking water promote productive performance via improvement of morphology in small intestine.

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Effect of indigenous lactic acid bacteria probiotics on poultry in Indonesia

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Introduction

The main pathogens associated with poultry farming and production are various serotypes of Salmonella enterica, Escherichia coli, and Campylobacter jejuni. These enteric pathogens are the most common causes of diarrhea in the poultry flock. Bacterial infection with extraneous pathogens can be avoided when poultry are reared intensively in good environmentally controlled housing and all appropriate biosecurity measures are followed. However, many small-scale farmers in Indonesia lack the knowledge and skill to deal with biosecurity measures often resulting in an outbreak of pathogen-associated disease and the death of birds. The main causes of pathogen spread are due to poor sanitation and drainage, as well as improper litter management, which all lead to bacterial infection in birds. As a solution, farmers usually utilize antibiotics as a growth-promoting feed supplement which is targeted either to eradicate bacterial infection or to promote the growth of birds by increasing the feed efficiency.

Antibiotics have been used for many decades by veterinarians and poultry farmers before they were banned in many countries due to concerns with increased antibiotic resistance in pathogens and antibiotic contamination in food. According to Jones and Ricke (2003), about 30 kinds of antibiotics are used in poultry feed as additives or administered as drugs, and it is estimated that more than 13.7 % of the antibiotics used in animal production are used at subtherapeutic (growth promoting) levels. The prohibition of antibiotic use is because the residues of these drugs present in meat and eggs constitute a potential health hazard to consumers. The major health risks associated with antibiotics are the development of antibiotic resistance in exposed individuals, hypersensitivity reactions, and the development of microorganisms resistant to antibiotics in humans (Dipeolu et al. 2005). When tetracycline is provided at 200 mg/kg feed to birds, it is eventually released in the eggs, while the metabolism of tetracycline continues in the body of the layer. Birds provided with an antibiotic-supplemented diet produced eggs with a tetracycline residue level of about 0.017 μg/g. These tetracycline residues started appearing in the egg from the second week after the birds were fed a tetracycline-supplemented diet. The tetracycline residues continued to present in the eggs even after 1 week of supplementation. The Codex's recommended maximum residue level (MRL) for tetracycline in eggs is 0.02 μg/g.

In the search for an alternative to antibiotics in poultry feed, the addition of probiotics has been proposed. Among all the probiotics utilized in poultry production, lactic acid bacteria (LAB) are the most commonly used. The addition of LAB replaces enteric pathogens by means of competitive exclusion in the poultry intestinal tract and subsequently reduces bacterial contamination in poultry products, thereby increasing the safety of chicken meat and eggs.

Characteristics of these indigenous lactic acid bacteria probiotics

Although probiotics are considered to promote poultry health, the actual mechanisms involved have not yet been fully elucidated. The most important advantage of probiotic application in poultry is that, unlike antibiotics, they leave no residues in the meats and eggs, which may have serious health implications for consumers.

Nowadays, it is well recognized that probiotics are strain-specific, live microbial cultures that produce beneficial effects on the host's body. These cultures could be a single species of bacteria or a combination of many microbes. They are commonly isolated from the digestive tract of a healthy adult animal, typically from the same species to which the probiotics will be administered. As an ideal probiotic, these bacteria should become a part of normal microbial flora in the intestine, survive gastrointestinal passage, and be able to adhere and colonize the intestinal tract.

Sri-Harimurti *et al.,* (2010) reported that indigenous LAB isolated from the digestive tract of healthy adult Indonesian native chickens (ayam kampung), including *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6, proved to be efficient as a feed supplement to improve the live performance of broiler chickens. After 35 days of treatment with a mixture of those probiotics the villi height, villi width, and crypt depth of the duodenum, jejunum, and ileum were statistically different ($P<0.05$) from the control group (unsupplemented with probiotics). It was showed that those probiotics supplementation in broiler chickens increases the villus height and villus width in all segmens (Sri-Harimurti *et al.*, 2013a,b). The increase was likely due to enhanced short chain fatty acid formation (ileal propionic and butyric acid) in male laying quils at 42 days of age (Sri-Harimurti *et al.,* 2014). Short-chain fatty acids (SCFAs), including propionic and butyric acids, are by-products of bacterial fermentation that stimulate the proliferation of the bowel epithelium. The most promising targets for probiotics are gastrointestinal functions, including those that control transit time, bowel habits, and mucosal motility as well as those that modulate the epithelial cells of the gastrointestinal tract, as presented in Fig. 2.

The in vitro adhesion assay showed that *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 had a good ability to adhere to IEC, as revealed by phase-contrast microscopy (Fig. 3). Scanning electron micrographs showed a clear appearance of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kp2, and *Pediococcus acidilactici* Kd6 attached firmly to the intestine of the supplemented broiler, but there was no attachment on the intestine of unsupplemented chickens (Fig. 4).

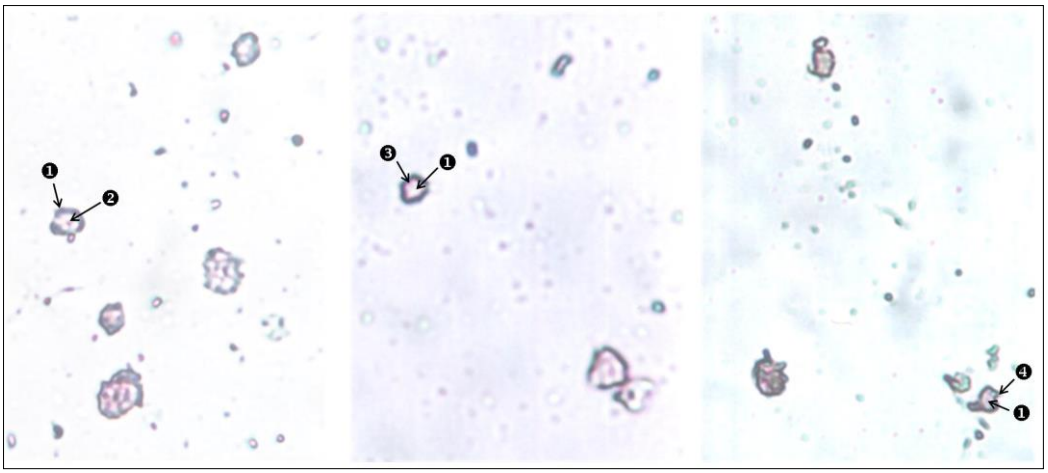

Fig. 3 Phase-contrast microscopy of adherence of lactic acid bacteria on intestinal epithelial cells (IEC) of the chicken. (1) IEC; (2) *L. murinus* (Ar3); (3) *S. thermophilus* (Kp2); and (4) *P. acidilactici* (Kd6)

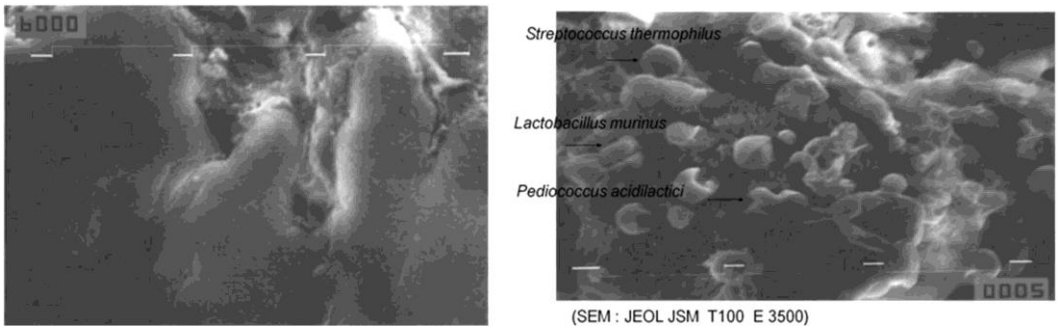

Fig. 4 Scanning electron micrograph of *Lactobacillus murinus* (Ar3), *Streptococcus thermophilus* (Kp2), and *Pediococcus acidilactici* (Kd6) in the chicken intestine. Left: unsupplemented chicken intestine; right: supplemented by those three strains. Scale bar ±1 μm.

Effect on broiler productivity and immune system

Application of a mixture of *Lactobacillus murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6 had significant effects on live weight, carcass yield, breast portion weight, abdominal fat weight, and abdominal fat percentage but not in carcass percentage when supplemented to broilers orally at 10^7 (T1), 10^8 (T2), and 10^9 CFU/ml/bird/day (T3). The carcass percentages in this experiment were 68.26±0.60 (T2), 68.66±1.86 (T3), and 69.20±0.90 (T1), similar to 70.40 observed with the application of a commercial Lactina probiotic. However, the live body weights with the supplementation of these probiotics 1824.50±38.89(T2),1899±43.50(T3), and 1966.20±38.05(T1) were higher compared to 1688.9 g in broilers supplemented with a commercial Lactina probiotic (Djouvinov et al. 2005a, b). The diameter of Peyer's patches in the intestine, as well as the weight of the bursa and spleen, as indicators of immune responses in chickens following these probiotics supplementation.

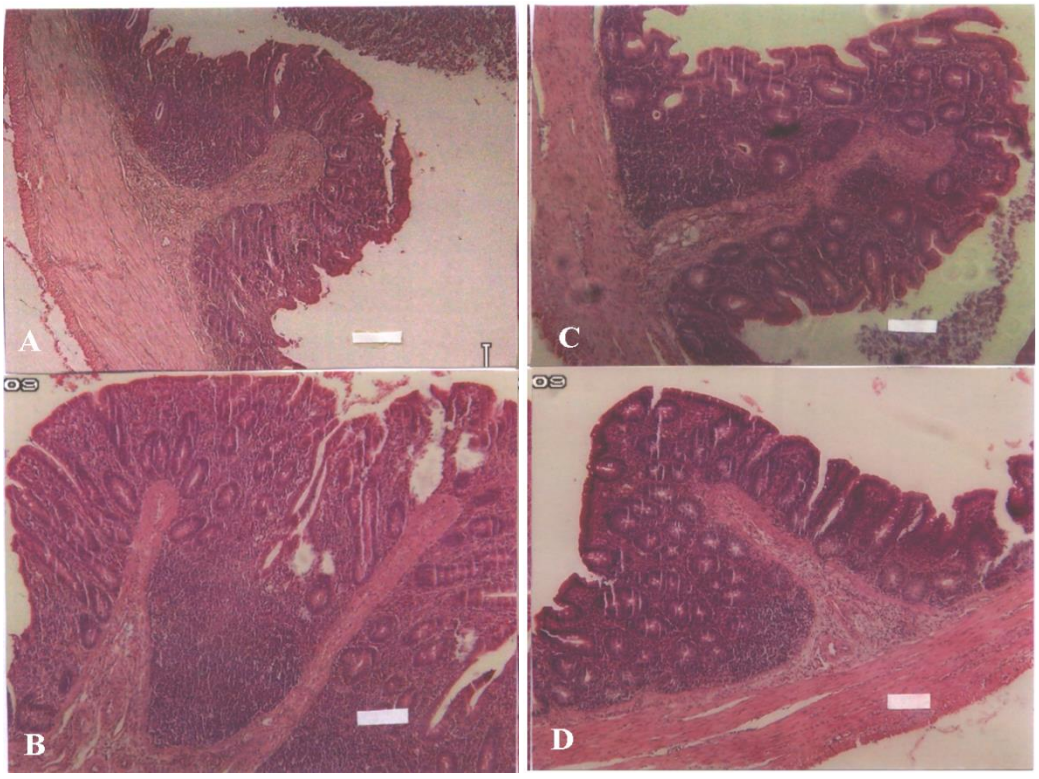

Fig. 2 Histology of the villi (ileum) of broilers after supplementation with probiotic LAB. Scale bar 10 µm. (A) Villi of control (unsupplemented); (B) supplemented with probiotics at 10^8 CFU/ml/chick/day; (C) supplemented with probiotics at 10^7 CFU/ml/chick/day; and (D) supplemented with probiotics at 10^9 CFU/ml/chick/day.

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Effects of probiotics on the innate immune-defense system by antimicrobial peptides in the gut mucosa of chicks

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Introduction

The mucosal of the gastrointestinal tract can be infected by various pathogenic microorganisms. The gut-associated lymphoid tissues have not been fully developed during the first week of life of chicks, whereas the immuno-protection could be provided during the first week of life through maternal antibodies and innate defense system. In contrast, innate immune system may play significant roles in the defense system since it is developed from early phase of life including the embryonic stage.

The antimicrobial peptides including avian β-defensins (*AvBD*s) and cathelicidins (*Cath*s) are the part of the main components involved in the innate immune-defense system. They demonstrate antimicrobial activities against a variety of microorganisms, including Gram-positive and Gram-negative bacteria as well as fungi and enveloped viruses. Fourteen *AvBD*s and 4 *Cath*s have been identified in chickens. Innate immune responses are stimulated by pathogen-associated molecular pathogens (PAMPs) via Toll-like receptors (TLRs). Chicken TLRs are important in the recognition of PAMPs to induce the production of pro-inflammatory cytokines and antimicrobial peptides. Ten TLRs have been reported in chickens (TLR1-type 1 and type 2 (1.1 and 1.2), 2.1 and 2.2, 3, 4, 5, 7, 15, and 21, and among them, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, and others also recognize their specific ligands of different viral and bacterial molecular patterns.

Probiotic may have beneficial effects on broiler performance as well as on the modulation of intestinal microflora and their genes (microbiome) to reduce pathogens. They have also been reported to modulate the expression of *AvBD*s in the mucosal tissue of the gut mucosa. Akbari et al. (2008) reported that the expression of *AvBD*s and cathelicidin was increased due to Salmonella infection in the chick intestines, but the administration of probiotics eliminated the effects of Salmonella infection on the expression of those antimicrobial peptides. Pro-inflammatory cytokines such as *IL1β*, *IL6*, *IFNγ*, and tumor necrosis factor super family 15 (*TNFSF15*) may cause not only the inflammation of tissues but also some of them may regulate the adaptive immune system and *AvBD*s expression as reported by the studies in the blood and reproductive tissues. If probiotics affect the expression of antimicrobial peptide and cytokines, they may be considered as the useful treatment to enhance the gut innate immune system.

In this paper, we describe the effects of probiotics on the expression of *AvBD*s, *Cath*s and cytokines in the gut challenged with or without LPS.

Effects of probiotics on the expression of AvBDs, Caths and pro-inflammatory cytokines in response to lipopolysaccharide stimulation in the proventriculus and cecum of broiler chicks

The aim of this study was to determine whether probiotic feeding affected the expression of *AvBD*s, *Cath*s and pro-inflammatory cytokines in response to lipopolysaccharide (LPS) challenge in the proventriculus and cecum of broiler chicks. One-day-old male Chunky broiler chicks were fed

with or without 0.4% probiotics for 7 days (P-group and non-P-group, respectively). Then, they were orally challenged with no LPS (0-LPS), 1 μg LPS (1-LPS), or 100 μg LPS (100-LPS) (n = 5 in all groups) in Experiment 1, and with no LPS and 1 μg LPS (n = 6 in all groups) in Experiment 2. Five hours after LPS challenge, the proventriculi and ceca were collected to analyze Toll-like receptors (*TLR*s), *AvBD*s, *Caths*, and *pro-inflammatory* cytokines expression by reverse transcription-PCR (RT-PCR) using 0-LPS chicks of experiment 1. The expressed TLRs, AvBDs, CATHs and cytokines were furtherly analyzed by quantitative real-time PCR in all groups in experiment 1 and 2. All *TLRs (TLR1.1, TLR1.2, TLR2.1, TLR2.2, TLR3, TLR4, TLR7, TLR15 and TLR21 and CD14* were expressed in the proventriculus and cecum of chicks. A total of 7 *AvBDs* (*AvBD1, 2, 4, 6, 7, 10,* and *12)* and *8 AvBDs* (*AvBD1, 2, 4-7, 10,* and *12)* were identified in the proventriculus and cecum, respectively. All four types of *Cath*s (*Cath1, 2, 3* and *4*) were expressed in the proventriculus and cecum of broiler chicks. Interleukin (*IL) 1β, IL6,* interferon *(IFN) γ* and *TNFSF15* were expressed in the proventriculus and cecum of chicks. In Experiment 1, the expression of 2 *AvBD*s in the proventriculus and 6 *AvBD*s in the cecum of 1-LPS chicks was higher in P-group than in the non-P-group. In Experiment 2, the expression of *AvBD1* in proventriculus and 5 *AvBD*s in cecum of 1-LPS- chicks was higher in P-group than in non-P-group. Challenge with 100-LPS did not cause differences in the *AvBDs* expression between P- and non-P-group. Expression of *Cath*s in cecum of 1-LPS- chicks was higher in P-group than in non-P-group. Although *IL1β* expression was not affected, the expression of *IL6* and *TNFSF15* in the proventriculus and expression of *IFNγ* in the cecum was lower in P-group than in non-P-group challenged with 100-LPS. These results suggest that probiotic feeding may enhance the immuno-defense system mediated by *AvBDs* and *Cath*s but not by cytokine, against infection by Gram-negative bacteria.

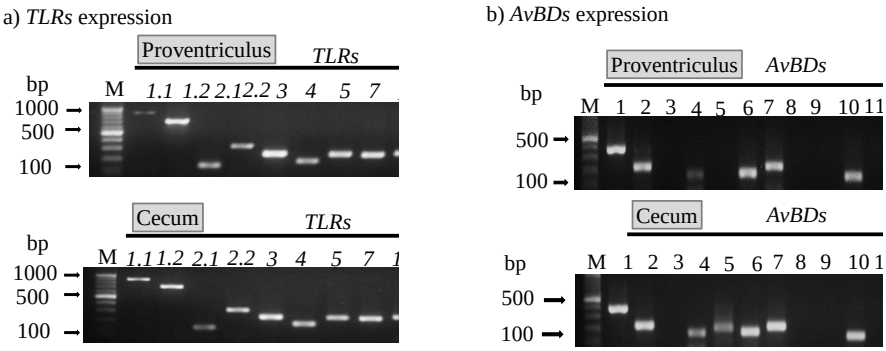

Fig.1 Expression of Toll-like receptors (TLRs) and avian β-defensins (AvBDs) in the chick gut.

Conclusion

In response to LPS stimulation the expressions of *AvBD*s and *Cath*s were higher in P-group than non-P-groups, suggesting that probiotics feeding either enhance the expression of these antimicrobial peptides or protect the reduction of their expression caused by LPS in the intestine. Thus, probiotics can protect the chicks through antimicrobial peptides immuno-modulatory effects.

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Introduction

We have shown that avian β-defensins (AvBDs) and cathelicidins are expressed in the gastrointestinal tract in chicks. The expression of AvBDs in response to LPS stimulation was higher in the chick fed with probiotics than those fed without probiotics. Akbari et al. (2008) also reported that the expression of AvBD1, AvBD2, AvBD4, AvBD6 was repressed by probiotics in combination with Salmonella infection. The expressed AvBDs may play roles in the defense against Gram-negative bacteria infection. Fourteen AvBDs have been identified in chickens till now. If the AvBDs expressed by the gut mucosa differ among the different breeds of chickens, it may affect the susceptibility of chicks. Hong et al. (2012) evaluated the changes in the expression levels of AvBD mRNA in necrotic enteritis disease model in 2 genetically disparate commercial broiler chicken lines: Ross and Cobb. They showed the differences in gene expression levels of AvBDs and proinflammatory cytokines in the intestine, crop, and spleen, suggesting the differences in their expression may affect the predisposed disease resistance and susceptibility to necrotic enteritis disease in the 2 commercial broiler chicken lines.

For better understanding the regulation of AvBD expression and synthesis, it is necessary to identify the cells responsible for the synthesis of AvBDs. Cuperus et al. (2016) reported that AvBD9 was predominantly found in enteroendocrine cells throughout the intestine, and cathelicidins-2 was exclusively found in heterophils in the several tissues including the intestine of embryonic and early posthatch chicks. They suggested that AvBD9 appears to be expressed in cell types strategically located to respond to infectious stimuli, suggesting these peptides play a role in the intestinal defense. However, the cells that synthesize other AvBDs remain unknown.

The aim of this study was to determine whether the expression profiles of AvBDs were different in different chicken breeds, and to identify the cells responsible for the synthesis of AvBDs in the chick intestine. We here examined the gene expression profiles of AvBDs in the gut of different chicken breeds, including Japanese native chicks and broiler chicks. Then, the intestinal cells immunoreactive for AvBD10 and 12 were identified.

Expression profiles of AvBDs in the gut of different breeds of chickens

The expression profiles of AvBDs in the proventriculus, ileum and cecum were compared among three Japanese native chicks, including Tosa-jidori, Hinai-dori, Oh-Shamo, and Chunky broiler chicks by RT-PCR. In the Chunky broilers, the expression of AvBD1, 2, 4, 7 and 10 in the proventriculus, AvBD1, 2 ,4, 7 and 10 in the ileum, AvBD1, 2, 4, 6, 7 and 10 in the cecum were identified. Most of these expression profiles were similarly observed also in the three Japanese native chicks, suggesting that AvBDs are expressed commonly in different breeds of chickens.

Identification of intestinal cells containing immunoreactive AvBD10 and 12

Sections of the ileum and cecum of chicks (0, 7 or 14-d-old) were immunostained using

rabbit anti-AvBD12 raised in our lab. Immunoreaction products of AvBD12 were identified in the epithelial cells of the crypts of both the cecum of the chicks.

Conclusion

We suggest there may be the polymorphism in the expression levels of AvBDs in the intestine at the individual level rather than breed levels when compared within Japanese native chicks. The epithelial cells of the crypts may be responsible to synthesize AvBD-10 and -12 in the ileum and cecum. Thus the ability to synthesize AvBDs in these cells may be considered for the strategy to enhance the resistance against infections in the chick intestine.

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