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Effect of Lactobacillin on the Mucosal Immune Function in Duodenum of Young Broiler

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Abstract: The experiment was conducted to investigate the effects of Lactobacillin in the mucosal immune function in duodenum of young broiler. A total of 300, 1 day old broiler chickens were randomly divided into five groups with four replicates per group and 15 chickens were in each replicate. The chickens in the control group (goup I) were fed with the basal diet, those in goup II were fed with basal diet supplemented with antibiotics and those in the rest groups (goups III-V) were fed with basal diet supplemented with 50, 100 and 200 mg kg^{-1} lactobacillin, respectively. The experiment was lasted for 28 days. The intestinal villi height, the number of mucilage cells, intraepithelial lymphocytes, mast cells and SIgA positive cells were detected by the technology of histochemistry and immunohistochemistry. The results showed that the height of intestinal villus in group II, IV was significantly more than group I ($p<0.01$) but there was not significantly difference between group II and IV. The results also showed that there was more mucopolysaccharide positive cells and mucilage cells in group II, group III and IV compared with group I ($p<0.05$). Compared with group I, the number of intraepithelial lymphocytes mast cells and SIgA positive cell in group II and IV have noticeably increased ($p<0.05$). It is concluded that Lactobacillin added to broiler diet with 100 mg kg^{-1} can improve the mucosal immune function in duodenum of young broiler.

Key words: Lactobacillin, broilers, mucosal immune, mucilage, China

INTRODUCTION

The application of sub-therapeutic antibiotics has been used as the feed additive for decades since, it has growth promotion function. And the antibiotics can change the microbiology flora of intestinal tract such as killing the enteropathogenic *Escherichia coli* (Manes and Batra, 2011). And the application of sub-therapeutic antibiotics has increased the economic benefits during the animal production which induce more and more farmer to use the antibiotic (Loh *et al*., 2010). However, the procedure cause public health consequences due to high risk of resistance of pathogenic bacteria in animal products. Therefore, it is highly demanded to find the ways of replacing antibiotics in animal feeding strategies. The emphasis of study has been on bacteriocins produced by lactic acid bacteria such as Lactococcus, Pediococcus, Lactobacillus and Carnobacterium in the past 30 years. The important reason for focusing on these bacteria is that they are traditionally used to produce fermented foods such as cheese and pickles. These products have been consumed by humans for countless generations with no evident health problems, consequently, these bacteria and their bacteriocins have an established reputation of safety. Lactic acid bacteria also have the distinction of producing the most commercial success and most studied bacteriocin to date, nisin (Garcera *et al*., 1993; Abee *et al*., 1994; Winkowski *et al*., 1994. Nisin is the premier example of a bacteriocin successfully developed for use as a food additive. It has a mass of approximately 3500 daltons and it is produced by some strains of *Lactococcus lactis* sub-species *lactis*. Nisin is effective against a wide number of Gram-positive bacteria. Nisin was first used as a preservative agent in 1951 and gained worldwide acceptance under the brand name, Nisaplin™. In the United States, it was first approved by the Food and Drug Administration as an additive in pasteurized processed cheese spreads in 1988. Nisin is currently the only bacteriocin licencedasa food additive that spreaded 45 countries (Delves-Broughton, 1990).

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Most bacteriocins from lactic acid bacteria are cationic, hydrophobic or amphiphilic molecules composed of 20-60 amino acid residues. These bacteriocins are commonly classified into three groups that also include bacteriocins from other Gram-positive bacteria. In class I, lantibiotics (from lanthionine-containing antibiotic) are small (<5 kDa) peptides containing the unusual amino acids, lanthionine, methyllanthionine, dehydroalanine and dehydrobutyrine. Class II contains small (<10 kDa), heat-stable and nonlanthionine containing peptides. The class III bacteriocins are not as well characterized as classes I and II (Klaenhammer, 1993).

The medicine function of nisin is antibacterium, improve the intestine condition and immune function. Though a lot of researches about Lactobacillin were carried out in resent years in which most focus on the selection of wide high-efficient antibacterial lactic acid bacteria (Van Reenen *et al*., 1998; Joerger and Klaenhammer, 1986) the application of it in farming was few reported.

The intestine contains functionally distinguishable populations of immune cells and neuropeptide-secreting cells which provide the anatomical basis for research on neuroimmune interactions (Ottaway, 1991; Genton and Kudsk, 2003). Oral immunization has received much attention in vaccine development because of its easy and safe administration. The immune cells in gut-associated lymphoid tissue lies in epithelial layer, mucosal lamina propria and mucosal lymphoid follicle. The intestinal epithelial cell, intraepithelial lymphocytes mast cell and SIgA-positive cell complete immune response together. The present study was designed to investigate effects of Lactobacillin on aspects of the mucosal immune function in duodenum of young broiler.

MATERIALS AND METHODS

Lactobacillin: Lactobacillin is the scientific achievements that is accomplish by Myron Biotechnology (Xiamen) Ltd. and College of Chemistry and Chemical Engineering of Xiamen University and have effective activematter 1.23×10^6 IU g^{-1}.

Experimental animal: A total 300, 1 day old healthy baby chicks were present by Guangdong Wens Food Group Co., Ltd.

Experiment design: A total of 300, 1 day old broiler chickens were randomly divided into five groups with four replicates per group and 15 chickens per replicate. The chickens in the control group (group I) were fed with the basal diet, those in group II were fed with basal diet supplemented with antibiotics and those in the rest groups (groups III-V) were fed with basal diet supplemented with 50, 100 and 200 mg kg^{-1} Lactobacillin, respectively. The experiment was lasted for 28 days. The basal diet was made up base on Table 1. The experiment lasted for 28 days.

Table 1: Composition and nutrient level of basal diet (air dry basis) (%)

Items	Content (0-28 days)
Ingredients	
Corn	52.30
Wheat middlings	10.00
Rice polishings	
Soybean meal	22.00
Peanut meal	3.00
Rice Bran meal	
Fishmeal (CP62%)	1.00
Rapeseed dregs	
Expanded soybean	8.00
Limestone	1.70
Premix[1]	2.00
Total	100.00
Nutrient levels	
ME (MJ kg^{-1})	12.08
CP (%)	20.13
Ca (%)	0.92
NPP (%)	0.30
Lys (%)	1.09
Met + Cys (%)	0.74

[1]The premix provides following per kg diet: Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 78 mg, Zn (as zinc sulfate) 60 mg, Mn 80 mg, I 0.4 mg, Se 0.2 mg, choline 800 mg, VA11250IU, VD_3 2500IU, VE 18.75 mg, VK_3 5 mg, VB_1 2.5 mg, VB_2 6.25 mg, VB_6 2.50 mg, VB_{12} 18.75 μg, nicotinic acid 25 mg, D-pantothenic acid 12.50 mg, Folic acid 1.25 mg, biotin 100 μg, 500 IU

Collection and management of samples: On the 28th day, two middleweight chicks that were pick out from every repeating group were decapitated. The duodenums were collected with the size of 1.5×1.5×0.5 cm and the intestinal tracts were clean out with physiological saline (pH = 7.1). Then, they were fixed in 4% paraformaldehyde fixing solution for 4 h at 4°C. Fallowed the method of routine histology, dehydration, transparence and embedding were completed. Samples were cut into 5 μm semiserial cross sections. Per tissue sample, one piece was picked up from each ten section, total 5 pieces from 50 sections.

Histochemical examination of mucosal immune cell: The height of intestinal villi and the number of intraepithelial lymphocytes were measured by hematoxylin and eosin stain and covered with coverslips.

Toluidine blue staining for mast cell: The staining fluid of toluidine blue was prepared according to Welle *et al*. (1995). About 1.0 g toluidine blue dissolved in 80 mL distilled water and 0.6 g of potassium permanganate dissolved in 20 mL distilled water. The toluidine blue solution was boiled 10 min and the potassium permanganate solution was added dropwise into it, then

the mixture solution was boiled 10 min sequentially. Finally, add the distilled water to 100 mL, let the mixed solution cooling and filter it, adjust pH to 1.0.

The sections were dewaxed and exchanged out of water by the gradient of ethanol on base of conventional histological methods, staining fluid solution cover on the section for 30 sec, washed two time with distilled water, 3 min per time. Color separation was done by 95% alcohol and control the separational time by the microscope. When the metachromatic granule in the cytoplasm of mast cell was showed fuchsia, the color separation was stopped. The sections were dehydrated by the gradient of ethanol and were transparent through dipping in the dimethylbenzene. At last, the sections were sealed with a glass coverslip. Tissues preserved in Carnoy's fluid and stained by toluidine blue revealed metachromatic character. The metachromatic granule in the cytoplasm of mast cell was showed fuchsia.

PAS stain for mucilage cell: The sections were dewaxed and exchanged out of water, oxidized 5 min by 0.5-1% periodic acid, washed in distilled water, stained 30-60 min at 37°C by Schiff, washed in sulfurous acid 2 times, washed in distilled water. At last, sections were cleared and sealed with a glass coverslip. Glycogen was shown fuchsia, glycoprotein was shown pink, mucin and mucopolysaccharide was shown red.

Immunohistochemical staining for SIgA-positive cells: Fifty sections at least 300 μm apart were prepared per sample. Endogenous peroxidase activity was inhibited by preincubating the tissues in 3% H_2O_2. The sections were then incubated in 5% normal goat serum for 0.5 h followed by an overnight incubation at 4°C with 1:40 dilution of rabbit antichicken IgA serum in PBS containing 0.02% Triton X-100 and 0.01% bovine serum albumin. After rinsing in PBS, sections were incubated with biotinylated goat antirabbit IgG (1:300, Vektor ABC kit, PK-6101) for 1 h at room temperature followed by incubation with an avidin-biotin-peroxidase conjugate solution for 1 h at room temperature. The sections were then rinsed 3 times with PBS and incubated with 3,3'-diaminobenzidin tetrahydrochloride (Sigma) solution dissolved in 0.05 M Tris-HCl buffer (pH 7.4) at room temperature. About 10 min later the enzyme-substrate reaction was stopped with 0.05 M Tris-HCl buffer (pH 7.4). Sections were then rinsed in PBS and counterstained with hematoxylin stain. At last, sections were cleared and sealed with a glass coverslip.

In the duodenum, IgA-positive lymphocytes were identified by their characteristic morphology: round with a nucleus surrounded by a ring of yellow-brown stain.

Analysis of tissue sections: Tissue sections were examined using the 40 stage objective of an LEICAR 2800 bright-field microscope, 5 microscope fields per section and 5 sections per tissue sample were examined. For each tissue section. The height of intestinal villi, the number of mucilage cells, intraepithelial lymphocytes, mast cells and SIgA positive cells in 5 microscope fields were counted and data expressed as the average number per microscope field. The datas were analyzed by one-factor analysis of variance of SPSS10.0 Statistical Software and done multiple comparison by LSD. Means were considered different at $p<0.05$ and very significantly different at $p<0.01$.

RESULTS AND DISCUSSION

Effect of lactobacillin on the height of intestinal villi in duodenum of young broiler: In the test, we found that the height of intestinal villi in group II was the tallest compare with other groups and height to 36.8% compared with group I ($p<0.01$). And the height of intestinal villi in the group IV height to 31.6% compared with group I ($p<0.01$). But the height of intestinal villi in the group IV was not significantly heighten compared with group II ($p>0.05$) (Fig. 1). It is thus clear that the height of intestinal villi in the young broiler fed with 100 mg kg^{-1} Lactobacillin was significantly heighten and the absorption area of duodenum was enhanced which were the same with fed with antibiotic.

Effect of Lactobacillin on duodenal mucous cells lever in duodenum of young broiler: The histochemica results was the glycogen was shown fuchsia, glycoprotein was shown pink, mucin and mucopolysaccharide was shown red (Fig. 2b). The number of three kinds of duodenal

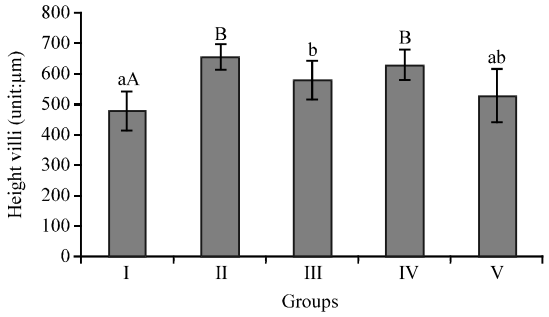

Fig. 1: Effect of Lactobacillin on the height of intestinal villi in duodenum of young broiler. The difference between data with the different small letter within a column is significant ($p<0.05$) and the difference between data with the different capital letter is very significant ($p<0.01$)

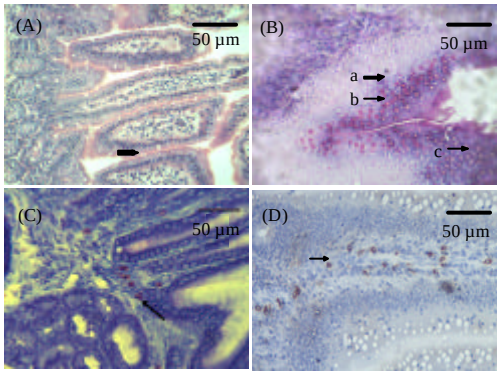

Fig. 2: Histochemical examination of mucosal immune cells in duodenum of young broiler. A) Hematoxylin and eosin stained section showing iIEL (viewed at 400x magnification); B) PAS stained section, a) shows Glycogen-positive cells, b) shows Mucin-positive cells and mucopolysaccharide-positive cells, c) shows Glycoprotein-positive cells; C) Toluidine blue stained showing mast cells; D) Immunohistochemical stained showing that SIgA-positive cells

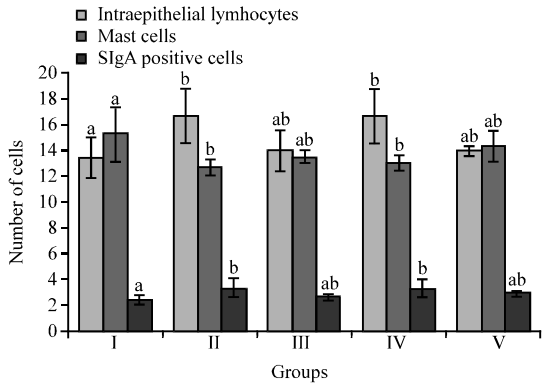

Fig. 3: Effect of Lactobacillin on intraepithelial lymphocytes, mast cells, SIgA positive cells in duodenum of young broiler

mucous cell was counted by using the 40 stage objective of an LEICAR 2800 bright-field microscope. In this test, researchers found that the number of mucopolysaccharide positive cell was the most in all groups but was not significantly increased compared with group II ($p<0.05$).

The total number of duodenal mucous cell was found the same result. So, the number of mucopolysaccharide positive cell in the young broiler fed with 100 mg kg^{-1} Lactobacillin was significantly heighten thus the total number of duodenal mucous cell was increased which lead to the mucus in duodenum was released in large amounts (Fig. 3). The young broiler fed with 100 mg kg^{-1}

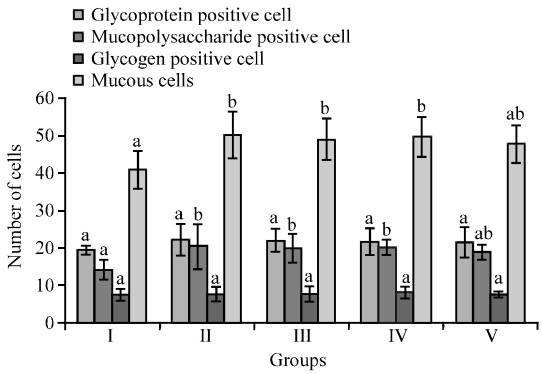

Fig. 4: Effects of lactobacillin on duodenal mucous cells in 28th broilers

Lactobacillin was significantly heighten in the young broiler fed with 100 mg kg^{-1} Lactobacillin was significantly heighten.

Effect of Lactobacillin on intraepithelial lymphocytes lever in duodenum of young broiler: The histological results showed that intestinal iIEL were localized between the cells of luminal epithelium layer and also in the basal region of the epithelium in the duodenum (Fig. 2a). There were more iIEL in group II and IV compared with group I ($p<0.05$) but the number of iIEL was not different between group II and IV (Fig. 4).

Effect of Lactobacillin on mast cells lever in duodenum of young broiler: The histological results showed that mast cells were localized in duodenum of broiler. Tissues preserved in Carrnoy's fluid and stained by toluidine blue revealed metachromatic character (Fig. 2c). The metachromatic granule in the cytoplasm of mast cell was showed fuchsia. In the test, we found that the number of mast cell in duodenum of group I was the most. And there were less mast cells in duodenum of group IV compared with group I ($p<0.05$). The difference was not observed between other groups ($p>0.05$) (Fig. 4).

Effect of Lactobacillin on SIgA positive cells lever in duodenum of young broiler: In the duodenum, SIgA-positive lymphocytes were identified by their characteristic morphology: round with a nucleus surrounded by a ring of yellow-brown stain. These cells were present in the lamina propria of villi in the duodenum (Fig. 2d). As shown in Table 4 there were more SIgA-positive cells in the duodenum in group II and IV ($p<0.05$) compared with the group I. The difference was not observed between other groups ($p>0.05$) (Fig. 3).

In most cases, bacteriocin production and activity has been demonstrated only in the laboratory. Evidence

for a role layed by bacteriocins in natural systems such as the intestinal tract is largely circumstantial. The observation that many intestinal bacteria such as *Fusobacterium mortiferum* isolated from chicken ceca are able to synthesize bacteriocins *in vitro* supports the notion that bacteriocins might be useful for survival in the intestinal tract (Hoover and Chen, 2010). Some data from experiments with bacteriocin-producing bacteria also suggest an influence of bacteriocins on the ecology of the intestinal microbiota. For example, an avian *Escherichia coli* strain genetically engineered to produce the bacteriocin microcin 24 lowered intestinal *Salmonella typhimurium* counts in chickens when administered continuously in the water supply (Wooley *et al*., 1999). Similarly, the bacteriocin-producing *Enterococcus faecium* strain J96 isolated from the crop of a chicken exhibited some protective effect on chicks infected with *S. pullorum* (Audisio *et al*., 2000).

The mucosa is the first line of defense against pathogenic microorganisms that enter the gastrointestinal tract (Yan *et al*., 2002). The intestinal villus is formed to increase the superficial area of intestinal tract. It is not only beneficial to the nutrient absorption but also can increase the mucosal immunity. In this test we found that the height of intestinal villi in group II which were fed with basal diet supplemented with 100 mg kg^{-1} lactobacillin was the tallest compare with other groups. Bacteriocins are ribosomally synthesized peptides exhibiting antibacterial activity in most cases, against bacteria closely related to the producer microorganism. Several bacteriocins from gram-positive bacteria display bactericidal activity with fairly broad inhibitory spectra and may be useful as antibacterial agents for various practical applications (Kayalvizhi and Gunasekaran, 2010). The bacteriocins from Lactic Acid Bacteria (LAB) have attracted significant attention because of their potential use as non-toxic and safe additives for food preservation and prevention of food spoilage by foodborne gram-positive pathogenic bacteria (Gao *et al*., 2010). And the bacteriocins from LAB can selectively kill intestinal bacteria, protect and promote the growth of probiotics, improve the intestinal microenvironment.

The epithelium of duodenum is consisted of goblet cells and columnar cells. They all can secrete mucus including mucin and mucooplysaccharide which form the mechanical barrier of duodenum mucosa to prevent the planting of pathogenic microorganisms. And the cell coat includes many kinds of carbohydrase and peptidase to enhance the digestion and absorption of saccharides and protein. In this test we found that the Lactobacillin could promote number of the mucin cells, especially the number of Mucopolysaccharide positive cell. Researchers considered that the Lactobacillin could promote the secrete of mucopolysacharide and against the planting of pathogenic microorganisms then enhance the mucosal immunity activity because the mucopolysacharide was found can stimulate immune system and antiviral.

It has been reported for various animal species that the characteristic, distribution and number of iIELs are dependent on mucosal immunization, suggesting that iIEL play a supporting role in the mucosal immune response It is well known that iIELs are programmed for cytokine production such as interleukin-IL-2, IL-5, Interferon-(IFN) α and IFN-β to protect against bacteria and viruses (Taguchi *et al*., 1991). The iIEL and Intestinal mucosa-associated lymphoid tissue together to complete the mucosa immune function. In this test, researchers suggested that the Lactobacillin could increase the number of iIEL to enhance the mucosa immune function of chicken.

SIgA was composed and secreted from the IgA positive plasma cells in the lamina propria of the intestinal mucosa. SIgA was released into enteric cavity and mixed with the normal flora. It can promote the settle of normal flora and inhibited the pathogen. Medici *et al*. (2004) fed BALB mice with Probiotic Fresh Cheese (PFC) and found PFC enabled *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. paracasei* to exert important immunomodulating effects in the gut (Medici *et al*., 2004).

CONCLUSION

In this study when we analyzed the effects of Lactobacillin administration on the number of IgA^+ cells, researchers observed that there was increasing number of IgA positive cells in group IV which was fed with 100 mg kg^{-1}. This might be due to This might be due to an autoregulation process that generally takes place once a maximal response is achieved.

The phenomenon would avoid an inflammatory response due to an overstimulation of the intestinal mucosa. The reduction in the number of IgA positive cells in group V compared with group IV was observed. Researchers believe that once a maximum response is achieved, it can last for a certain period of time. The length of this response depends on the length of the production of the cytokines implied in the clonal expansion of B cells. However, if the stimulation last for two long and to avoid an inflammatory response, a down-regulation phenomenon might be induced.

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