Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Jan - Mar 2017 / v.19 / n.1 / 115-122

http://dx.doi.org/10.1590/1806-9061-2015-0180

Bacillus Coagulans Enhance the Immune Function of the Intestinal Mucosa of Yellow Broilers

Author(s)

Xu L[I]
Fan Q[I]
Zhuang Y[I,II]
Wang Q[I,II]
Gao Y[I]
Wang C[I]

[I] College of Animal Sciences – Fujian Agriculture and Forestry University, Fuzhou 350002, China.
[II] Fujian Key Laboratory of Traditional Chinese Veterinary Medicine and Animal Health (Fujian Agriculture and Forestry University)

Mail Address

Corresponding author e-mail address
Quanxi Wang
College of Animal Sciences – Fujian Agriculture and Forestry University, Fuzhou 350002, China.
Tel: (+086)059183758852
E-mail: wqx608@126.com

Keywords

Bacillus coagulans, yellow broilers, growth performance, intestinal mucosa immune function.

Submitted: August/2016
Approved: November/2016

ABSTRACT

This experiment was conducted to investigate the effects of *Bacillus coagulans* on the growth performance and immune functions of the intestinal mucosa of yellow broilers. Three hundred and sixty one-day-old yellow chicks were randomly allocated to four treatments groups with six replicates of 15 chicks each. The broilers were randomly subjected to one of the following treatments for 28 days: control group (group1, fed a basal diet) and three treatments (group 2, 3, 4) fed the basal diet supplemented with 100, 200, or 300 mg/kg *Bacillus coagulans*, respectively). The results showed that for 28 days, compared with the control diet, the dietary addition of 200 mg/kg *Bacillus coagulans* significantly decreased the feed/gain ratio (F/G) ($p<0.05$), improved the thymus index, spleen index and bursa index ($p<0.05$), increased the villus height to crypt depth ratio (V/C) in the duodenum ($p<0.05$), increased the number of secretory immunoglobulin (sIgA) positive cells *(*$p<0.05$*).* The dietary addition of 200 mg/kg *Bacillus coagulans* promoted a significant increase in *Lactobacillus* spp. populations and suppressed *Escherichia coli* replication in cecum, compared with the control ($p<0.05$). Moreover, the dietary addition of 200 mg/kg *Bacillus coagulans also* significantly enhanced the levels of interferon alpha (IFNα), toll-like receptor (TLR3), and melanoma differentiation-associated protein 5(MDA5) in the duodenum ($p<0.05$). In conclusion, the dietary addition of *Bacillus coagulans* significantly improved broiler performance, and enhanced the intestinal mucosal barrier and immune function. The optimal dosage of *Bacillus coagulans* for yellow broilers was determined as 2×10^8 cfu/kg.

INTRODUCTION

Moore firstly reported that the animal weight was significantly enhanced by the addition of antibiotics in the feed in 1946 (Moore *et al.*, 1946). In the 1950s, the Food and Drug Administration (FDA) of the United States firstly approved antibiotics as feed additives, after which they were widely applied in the poultry industry for the treatment and prevention of bacterial diseases and as growth promoter. However, the negative effects of the extensive use – and even abuse – of antibiotics gradually emerged, such as antibiotic residues in meat (Smither *et al.*, 1980), bacterial resistance (Krushna Chandra Sahoo *et al.*, 2010), intestinal flora imbalance, and environmental pollution (Pan *et al.*, 2011), leading several countries to introduce legislation to limit the application of antibiotics in animal feeds.

The ban on the use of antibiotics as feed additives is an inevitable trend in China, and therefore, the research on alternatives for in-feed antibiotics is urgent. At present, there are many studies on such alternatives, such as beneficial bacteria, prebiotics, enzymes, acidulants, and plant extracts (Vahjen *et al.*, 2007; Natsir *et al.*, 2010). Probiotics maintain intestinal flora balance, enhance the intestinal barrier function (Anderson *et al*., 2010; Dai *et al*., 2012; Furrie E., 2005), and the immune function. Probiotics also can effectively improve the activity of interferon, which stimulates the immune cells to produce specific antibodies, such as sIgA, improve the discrimination of immune system, and induce cytokine expression in T and B lymphocytes and macrophages (Russell *et al*., 2013). Rajput reported that the dietary inclusion of a yeast (*Saccharomyces boulardii*) and *Bacillus subtilis* significantly increased the weights of the bursa and the thymus, and increased the mRNA expression levels of the occluding, cloudin 2 and cloudin 3 nucleotides, the number of IgA-positive cells in the jejunum, as well as the intestinal levels of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF) alpha, transforming growth factor beta (TGFβ), and sIgA (Rajput *et al.*,2013).

Intestinal-related immune system can identify gut microbes by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) (Gómez-Llorente *et al*., 2010). Probiotics can activate the mitogen-activated protein kinase (MAPK) and nuclear factor kappa(NF-kB) to activate TLRs, thereby regulating the immune function (Lebeer *et al*., 2010; Kawai *et al*., 2010; Wells *et al*., 2011). Probiotics can also regulate the immune function by regulating the inflammatory reaction (Castillo *et al*., 2011).

In this experiment, the effect of Bacillus coagulans on the growth performance and the immune function of the intestinal mucosa of yellow-feathered broilers was investigated.

MATERIALS AND METHODS

Birds and management

A total of 360 one-day-old healthy *Lingnan yellow-feathered chickens* were provided by Guangdong Wens Food Group Co., Ltd.

The experiment was performed at the poultry laboratory of Fujian Agriculture and Forestry University in Fuzhou, Fujian province, China. Fifteen chickens were reared per cage equipped with a drinker and a feeder. The water changed and feed was added to the feeders once daily. Birds were submitted to 23 hours of light. Vaccination was carried out according to Table 1.

Bacillus coagulans powder, containing that contained 1×10^{9}cfu/g living bacteria, was provided by Luodong Bio-Technology CO. LTD.

Experiment design

Chickens were randomly divided into four treatments with six replicates of 15 birds each. The chickens in the control group (group 1) were fed with a basal diet, and those in other groups (groups 2, 3, 4) were fed with the basal diet supplemented with 100, 200, or 300 mg/kg *Bacillus coagulans*, respectively. The experiment lasted for 28 days.

The basal diet was formulated according to China's poultry industry standards for Chinese color-feathered chicken between 1 to 28 days old. The diet was formulated for the entire period and supplied as mash. The *Bacillus coagulans* powder was added in the premix. The composition of the basal diet is shown in Table 2.

Table 2 – Composition and nutrient level of basal diet (air dry basis) %

Items	Content(0-28d)%
Corn	58
Soybean meal	27
Expanded soybean	10
Limestone	1.0
Premix^1	4.0
Total	100.0
Nutrient levels	
Metabolizable Energy (ME), MJ·kg^{-1}	11.87
Crude Protein (CP), %	21.20
Calcium (Ca), %	1.02
Total Phosphorus (TP), %	0.55
Available Phosphorus (AP), %	0.31
Lysine (Lys), %	1.12
Methionine (Met) + Cystine (Cys), %	0.83

1) The nutrient values in the table are calculated values.

2) The premix supplied per kg diet: Cu (as copper sulfate) 10mg, Fe (as ferrous sulfate) 72mg, Zn (as zinc sulfate) 60.2mg, Mn (manganese) 78mg, I (iodine) 0.4mg, Se (selenium) 0.24mg, choline 600mg, Vit. A (Vitamin A) 10000IU, Vit. D_3 (Vitamin D_3) 2600IU, Vit. E (Vitamin E) 26mg, Vit. K_3 (Vitamin K_3) 2.6mg, Vit. B_1 (Vitamin B_1) 2.6mg, Vit. B_2 (Vitamin B_2) 6.5 mg, Vit. B_6 (Vitamin B_6) 2.60mg, Vit. B_{12} (Vitamin B_{12}) 19.5μg, nicotinic acid 26mg, *D*-pantothenic acid 13mg, Folic acid 1.3mg, biotin 104μg, methionine 2364IU.

Table 1 – The immune program

Age	Vaccine	Dose	Immune method
1 day old	Marek's disease vaccine Combined vaccination of Newcastle disease and infectious bronchitis	One feather One feather	Intramuscular injection Eye droppings
5 day old	Avian influenza vaccine Fowlpox vaccine	One feather One feather	Intramuscular injection Hypodermic injection
10 day old	Newcastle disease vaccine	One feather	Intramuscular injection
20 day old	infectious bursal disease vaccine Newcastle disease vaccine Avian influenza vaccine	One feather One feather One feather	eye droppings Intramuscular injection Intramuscular injection

Growth performance parameters

Chicks were individually weighed on day 1 of the trial, and no significant differences were detected. On day 28, chicks were fasted for 12h and individually weighed. Feed was offered daily at 5:00 pm, after feed residues were measured. Average daily feed intake, average daily weight gain, and feed to gain ratio were calculated per experimental group. Livability (%) was calculated weekly.

Immune organ index

On day 28, three chicks in per replicate were selected and sacrificed by decapitation. The thymus, spleen and bursa were collected and weighed. Thymus, spleen, and bursa indexes were calculated as = [organ weight (g)/ body weight (g)] ×100.

Bacterial flora detection in the cecum by SYBR-PCR

Standard curve preparation

Plate count: Escherichia coli strain k88 was used as reference strain. It was cultured in lysogeny broth (LB) at 37 ^{o}C for 7 h, and then was serially diluted to 10^{-9}. Dilutions were then cultured in Macconkey medium at 37^{o}C for 24 h. Finally, bacterial colonies were counted.

Standard curve: The DNA of *Escherichia coli* strain k88 was serially diluted to $10^{-6.}$, and was detected by Quantitative Real-time Polymerase Chain Reaction (SYBR-PCR), and the linear equation was calculated.

Detection of Escherichia coli and Lactobacillus spp.

The universal primers of bacterial genera and the specific PCR primers of *Escherichia coli* and *Lactobacillus* genus were designed (Table 3). On day 28, the cecal content (200 mg) from the three chicks per replicate sacrificed for organ index were collected, and total DNA was extracted using a genomic DNA extraction kit (Taingen, Beijing, China). *Escherichia coli* and *Lactobacillus* spp. in the cecal content were detected by SYBR-PCR.

Reaction volumes of 25 μL consisted of 12.5 μL SYBR® Premix Ex TaqTM (2×) (Promega, Wisconsin, U.S.A), ROX reference dye (50×) 0.5 μL, 1 μLDNA, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), and 10 μL RNase free ddH_2O. PCR conditions were initial denaturation at 95^{o}C for 0.5 min followed by 40 cycles at 95^{o}C for 5 s, 60^{o}C for 34 s, and 95^{o}C for 15 s, 60^{o}C for 1 min, 95^{o}C for 15 s. Data was calculated by delta delta CT(△△CT), and were analyzed by SPSS19.0.

Villus height / crypt depth ratio

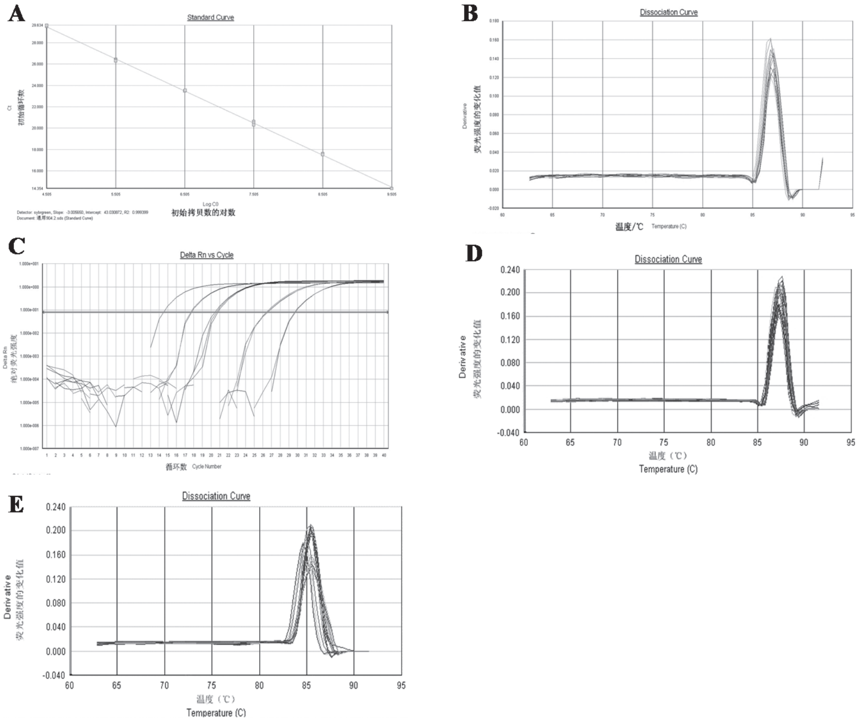
On day 28, three birds per treatment were sacrificed for organ index determination, and the intestinal contents were rinsed with physiological saline (PBS, 0.01mol/L, pH=7.1). Duodenal sections (1.5cm×1.5cm×0.5cm) were collected, fixed in 4% paraformaldehyde fixing solution for 24 hours, and were submitted to routine histology procedures, including dehydration, clearing, and embedding in paraffin. Samples were cut into 4 to 6μm semi-serial cross sections and stained with hematoxylin and eosin (HE). Intestinal villus height and crypt depth were measured under an optical microscope at 40x magnification using the software HPIAS-5100 (Qianping, Shanghai, China).

Detection of sIgA by immunohistochemistry

The sections of duodenum from the chicks sacrificed for organ index were prepared and inhibited by preincubating the tissues in 3% H_2O_2, incubated in 5% normal goat serum for 0.5h followed by an overnight incubation at 4^{o}C with 1:40 dilution of rabbit anti-chicken IgA serum. Sections were then incubated with goat anti-rabbit IgG (1:300, Vektor ABC kit, PK-6101) at room temperature for 1h, followed by incubation with an avidin-biotin-peroxidase conjugate solution at room temperature for 1h. The sections were then rinsed three times with phosphate salt buffer (PBS) and were incubated with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, Calif, U.S.A) solution dissolved in 0.05 *M* Tris-HCl buffer (pH 7.4) at room temperature.

Table 3 – Primer sequences

Items	Primer sequences	Amplified fragments(bp)
Bacterium universal primer (16s)	F: 5′-CCTACGGGAGGCAGCAG-3′ R: 5′-ATTACCGCGGCTGCTGG-3′	194
Escherichia coli(16s)	F: 5′-GTTAATACCTTTGCTCATTGA-3′ R: 5′-ACCAGGGTATCTTAATCCTGTT-3′	340
Lactobacillus genus(16s)	F: 5′- AGCAGTAGGGAATCTTCCA-3′ R: 5′ -CACCGCTACACATGGAG -3′	341
IFN-α	F: 5′ -GGACATGGCTCCCACACTAC-3′ R: 5′ -ATCCGGTTGAGGAGGCTTT-3′	204
TLR3	F: 5′ -CCATTTGATTGCACCTGTGA-3′ R: 5′ -GCAACACCAGAGTACCGTGA-3′	133
MDA5	F: 5′ -GAAGAAGGTGTCCGCTTATCA-3′ R: 5′ -GAATCTGAGGCTGTGGAATCA-3′	169
β-actin	F: 5′ -CCAAAGCCAACAGAGAGAAGAT-3′ R: 5′ -CATCACCAGAGTCCATCACAAT-3′	138



A: Primer specificity identification showed that the designed primers can be used for real-time fluorescence quantitative PCR reaction. (1: Escherichia; 2: Negative control of Escherichia; 3: Lactobacillus; 4: Negative control of Lactobacillus; M: 2000 DNAmarker)

B: Standard curve of standard samples (Escherichia Coli. strain k88 DNA)

C: Melting curve of standard sample

D: Amplification curve of standard sample

E: Melting curve of E. coli

F: Melting curve of Lactobacillus

Figure 1 – Bacillus coagulans optimized the bacterial flora in the yellow feather broiler chickens

Ten minutes later the enzyme-substrate reaction were stopped with 0.05*M* Tris-HCl buffer (pH 7.4). Sections were then rinsed in PBS and counterstained with hematoxylin. Finally, 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 yellow-brown stained ring.

Expression of INF, TLR-3 and MDA-5 mRNA

Quantitative real-time PCR was performed with the primers shown in Table 3 to analyze the mRNA expression levels of interferon (IFN) and pattern recognition receptors (TLR-3, MDA-5) to verify if the *Bacillus coagulans* product stimulated interferon production to enhance intestinal mucosal immune function. Data was calculated and analyzed by △△CT.

RESULTS

Growth performance

Table 4 shows the performance results obtained during the 28 days of the experiment. Average daily feed intake was not different among treatments (p>0.05). Average daily weight gain was 0.40% (p>0.05), 3.75% (p<0.05), 3.23% (p>0.05) higher in groups 2, 3, and 4, respectively, compared with group 1. Average body weight gain of group 3 was 3.73% higher than in group 1 (p<0.05). The feed conversion ratio of group 3 was 2.55% lower than that of group 1 (p<0.05). Compared with group 1, groups 2, 3 and 4 presented 1.15%, 1.15%, and 2.30% survival rates, but the difference was not significant (p>0.05). These results showed that the growth performance of chickens fed with 200 mg/kg *Bacillus coagulans* was significantly enhanced.

Immune organ index

Immune organ index indicates status of the avian immune function. As shown in Table 5, the thymus index of groups 2, 3, and 4 was 7.18% (p<0.05), 7.27% (p<0.05), and 0.56% (p>0.05) higher compared with group 1. The spleen index of groups 2, 3, and 4 was 2.75% (p<0.05), 14.66% (p<0.05), and 10.91% (p>0.05), respectively, higher than that of group 1. The bursa index of groups 2, 3, and 4 was 14.24%, 14.28%, 14.24% (p<0.05) than that of group 1. These results confirm that the dietary inclusion of 100 and 200 mg/kg *Bacillus coagulans* can significantly increase the immune organ index of chickens, particularly at 200 mg/kg.

Table 4 – Effects of *Bacillus coagulans* on the growth performance in yellow broilers

Treatments	Average daily feed intake	Average daily weight gain	Feed conversion ratio	Survival rate
1	29.75±0.81	15.18±0.46^{b}	1.96±0.04^{a}	96.67±3.65
2	29.70±0.68	15.24±0.30^{ab}	1.95±0.05^{ab}	97.78±3.44
3	30.14±0.82	15.75±0.44^{a}	1.91±0.02^{b}	97.78±3.44
4	30.52±0.85	15.67±0.48^{ab}	1.95±0.02 ^{ab}	98.89±2.72

Means followed by different superscripts in the same column are statistically different (*p*<0.05).

Table 5 – Effects of *Bacillus coagulans* on the immune organ index (%) of yellow broilers

Treatments	Thymus index	Spleen index	Bursal index
1	0.4292±0.0435^{a}	0.1623±0.0216^{a}	0.2844±0.0466^{a}
2	0.4600±0.0246^{b}	0.1830±0.0236^{b}	0.3249±0.0345^{b}
3	0.4604±0.0277^{b}	0.1861±0.0264^{b}	0.3250±0.0517^{b}
4	0.4316±0.0393^{ab}	0.1800±0.0194^{ab}	0.3249±0.0432^{b}

Means followed by different superscripts in the same column are statistically different ($p<0.05$).

Cecal bacterial flora

Plate count results showed that the number of *Escherichia coli* strain k88 in the cecum was 1.6×10^{10} cfu/mL. The regression coefficient (R^2) of the curve was 0.9994, indicating that the counts were linearly correlated with the dietary inclusion levels of the product. The equation of the standard curve was $y=-3.006x+43.031$ (y: Ct value’x: log value of standard DNA samples).

Total bacterial counts in three groups fed with three different doses *Bacilllus coagulans* were not different ($p>0.05$) compared with the control group (Table 6). *Escherichia coli* counts in the cecum of broilers were not significantly different among treatments ($p>0.05$). However, *Lactobacillus* spp. counts were significantly increased in the cecum of broilers fed with 200 mg/kg *Bacillus coagulans* in basal diet ($p<0.05$).

Table 6 – Effects of *Bacillus coagulans* on the microbial flora ($\lg\text{cfu}\cdot\text{g}^{-1}$) of yellow broilers.

Treatments	Total bacterial count	*Escherichia coli*	*Lactobacillus* spp.
1	11.85±0.21	10.67±0.38	9.34±1.14^{a}
2	11.87±0.13	10.36±0.32	9.73±1.16^{ab}
3	11.88±0.22	10.42±0.41	10.18±0.76^{b}
4	11.84±0.19	10.37±0.41	9.40±0.78^{ab}

Means followed by different superscripts in the same column are statistically different ($p<0.05$)

Duodenal villus to crypt ratio

On day 28, intestinal villus height in three groups fed with three doses of *Bacilllus coagulans* was not different change compared with the control group ($p>0.05$), but the duodenal crypts of group 3 were significantly deeper compared with the control group ($p<0.05$). Therefore, the villus height to crypt depth ratio (V/C) value was higher in the duodenum of broilers fed with 200 mg/kg *Bacillus coagulans* in the basal diet (Table 7).

Table 7 – Effects of *Bacillus coagulans* on the villus height to crypt depth ratio (V/C value) of duodenum in yellow broilers.

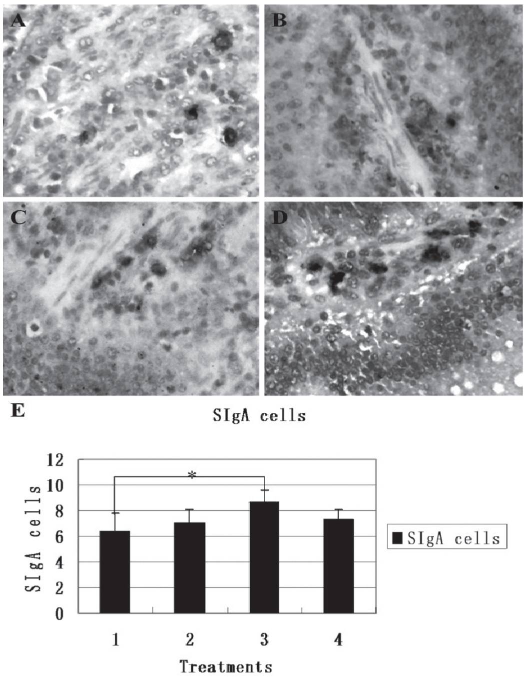
Treatments	*Intestinal villi* height (μm)	Crypt depth (μm)	V/C value
1	1120.91±41.24	114.48±8.54^{a}	9.83±0.78^{a}
2	1157.63±35.33	108.93±7.75^{ab}	10.68±0.96^{ab}
3	1160.20±45.00	103.39±9.19^{b}	11.30±1.11^{b}
4	1134.50±28.22	105.79±7.54^{ab}	10.76±0.72^{ab}

Means followed by different superscripts in the same column are statistically different ($p<0.05$)

Levels of sIgA-positive cells lever *in duodenum*

In the duodenum, sIgA-positive lymphocytes were identified by immunohistochemistry. These cells were present in the lamina propria of duodenal villi (Fig. 2 A-D). Fig. 2 E shows that there were more sIgA-positive cells in the duodenum of group 3 ($p< 0.05$) compared with group 1. No differences were observed between the other groups ($p>0.05$).

mRNA expression of IFN-α, MDA-5 and TLR-3

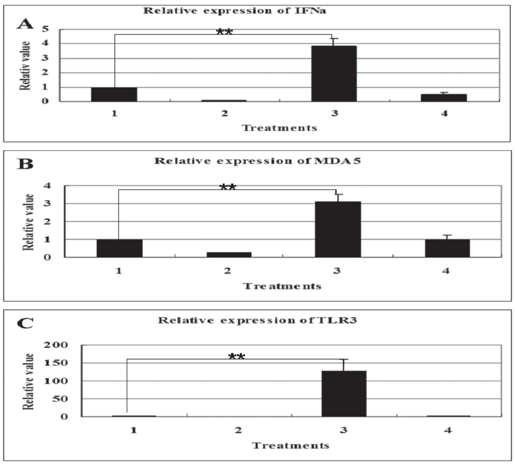


A-D: SIgA-positive lymphocytes were identified by immunohistochemistry method with characteristic antibody. These cells were present in the lamina propria of villi in the duodenum. A: group I, B: group II, C: group III, D: group IV. E: Statistical analysis on the number of SIgA-positive cells. *: $p< 0.05$.1-4: group I-IV.

Figure 2 – Bacilllus coagulans increase SIgA-positive cells in the duodenum

IFN-α is an important immune regulator, and may stimulate the innate immune and antiviral responses.

The objective of this measurement was to investigate if the *Bacilllus coagulans* was able to regulate the innate immune function of the intestinal tract. The results showed that, on day 28, the mRNA expressions of IFN-α, MDA-5 and TLR3 were significantly higher in group 3 compared with group 1 ($p<0.05$ or $p<0.01$) (Fig. 3), indicating that *Bacilllus coagulans* may stimulate the innate immune function of the intestinal tract.



A: IFN- α, B: MDA5, C: TLR3, 1: group I, 2: group II, 3: group III, 4 group IV

Figure 3 – Bacilllus coagulans promoted the mRNA expression of IFN-α, MDA5 and TLR3

DISCUSSION

The results of this experiment showed that the growth performance of chickens fed 200 mg/kg *Bacilllus coagulans* was effectively improved, which is consistent with the other reports. Lei reported that the dietary inclusion of spores of a lactobacillus improved the conversion ratio and reduced mortality and culling rate of broilers (Lei *et al*., 2015). Lactobacilli improve calcium and phosphorus utilization, and lactic acid promotes the transformation of pepsinogen into protease, stimulating the peristalsis of the small intestine to enhance nutrient digestion (Ma *et al.,* 2014; Giang *et al*., 2010).

Thymus, spleen, and bursa of Fabricius are important immune organs of poultry, and their index reflects the immune function of the poultry. Our results are in agreement with the findings of Jin Er-hui (2013), who showed that the 21-day old and 42-day old AA chickens fed] a bacillus-based probiotic contained 1x10^{11}cfu/g provided by Jiaxing Kori Biological Technology Co., LTD at 200 mg/kg presented better development of the immune organs and immune function than 400 mg/kg (Jin *et al*., 2013). Other studies have shown that *Bacillus* spp. regulate the intestinal bacterial flora and improve the immune function of the intestinal mucosa (Isolauri *et al*., 2001).

The ceca are the main site of intestinal microbial replication and activity. *Lactobacillus* spp. and bifidobacteria are the predominant genera (Apajalahti *et al*., 2004). These are commensal bacteria and can suppress the harmful bacteria and inflammation (Sheil *et al*., 2007). *Escherichia coli* is a conditionally pathogenic bacterium, and may become harmful and cause diarrhea when the intestinal microbial flora is imbalanced (Zhang *et al*., 2010).

Bacillus coagulans is able to produce bacteriocins, such as lactosporin, which have significant antibacterial activity (Riazi *et al*., 2009). In addition, it produces lactic acid and other organic acids, reducing the colonization of harmful bacteria in the intestinal tract (Cui *et al.*, 2005). *Bacillus coagulans* also breaks down polysaccharides into oligosaccharides, promoting the growth of lactobacilli, bifidobacteria and other beneficial bacteria, maintaining intestinal flora balance (Zheng *et al*.,2011). In recent years, a new compound, dysprosium, produced from *Bacillus coagulans* was shown to have broad antibacterial spectrum and to be highly efficient (Honda *et al*., 2011). Studies found that morphological changes in the small intestine were closely related to a toxin produced by enterotoxigenic *Escherichia coli* (ETEC) in the gut Jindal *et al.*,2006.

The small intestine is the main organ for the digestion and absorption of nutrients. Villus height, crypt depth and V/C ratio are indication of nutrient absorption capacity by the intestine (Caspary *et al*., 1992). In the present experiment, there was a positive and significant correlation between the intestinal villus height and the number of epithelial cells. Increases in the number of intestinal epithelial cells and in villus height indicates better nutrient absorption capacity ability of the body enhanced. Crypt depth reflects the secretory function of small intestine. *Bacillus* spp. can stimulate the differentiation and proliferation of intestinal epithelial cells and improve nutrient utilization (Artis *et al*., 2008; Wells *et al*., 2011; Duerr *et al*., 2012). Lei reported that broilers receiving a direct-fed microbial based on *Bacillus amyloliquefaciens* significantly increased crypt depth and villus height to crypt depth ratio in the duodenum, jejunum, and ileum (Lei *et al.,* 2015). Similarly, in broilers fed diets with 200 mg/kg *Bacillus coagulans*, an increase in V/C ratio was also observed (Lin *et al*., 2014).

The function of the intestinal barrier is to maintain epithelial integrity and to protect the body from the environment. The intestinal barrier functions include the mucous layer, secretory IgA and epithelial junction adhesion complex (Miriam *et al.*, 2012). If this barrier is disrupted, antigens reach the submucosa and induce inflammatory response (Wang *et al.*, 2012). The integrated intestinal mucosa barrier is important for the defense against pathogenic bacteria (Blikslager *et al.*, 2007).

Secretory IgA is produced and secreted by IgA-positive plasma cells in the lamina propria of the intestinal mucosa. It is released in the intestinal lumen and mixed with the normal flora. It allows the establishment of the normal flora and inhibit pathogen colonization. Medici fed BALB mice with probiotic fresh cheese (PFC) and found the PFC enabled *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. paracasei* to exert important immunomodulating effects in the gut (Medici *et al.*,2004). In our study, the number of IgA-positive cells was significantly increased when broilers were fed 200 mg/kg *Bacillus coagulans*, suggesting that this probiotic may enhance the immune function of the intestinal mucosa.

The intestinal mucosa is rich in lymphoid tissues, called the gut-associated lymphoid tissue (GALT). GALT can secret multiple cellular factors, such as interferon, colony stimulating factors (CSFs), interleukins, etc., which are able to kill pathogens and regulate the immune function of the mucosa. Interferons, in particularly, mediate the innate immune. Interferons are produced in response to pathogen infection. However, the infecting pathogen need to be recognized by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), melanoma differentiation-associated protein 5 (MDA-5)-like receptors, increasing the expression of IFN type I in the infected cells. MDA5 is one of the most important PRRs (Durbin *et al.*,2013). MDA-5 plays the critical role in the recognition of pathogens in the cytoplasm and transmits a signal to induce expression of interferon and cytokines. The newly-synthesized IFN-I is then secreted and binds to the IFN-I receptor (IFNAR), inducing the expression of hundreds of IFN stimulating genes (ISGs) that promote immune regulation.

Kailova reported that mice suffering from necrotizing enterocolitis (NEC) were orally received *Bifidobacterium* OLB 6378, which stimulated the mRNA expression of TLR2, cyclo-oxygenase2 (COX-2), and increased the synthesis intestinal of prostaglandin estradiol 2 (Kailova *et al.*,2010). Liu also verified that Roy's lactobacillus DSM 17938 rat NEC has a positive effect on the prevention on rat NEC, which can significantly reduce the mRNA expression of TNF alpha and TLR 4 and TNF alpha, TLR 4 protein levels (Liu *et al.*, 2012). Rajput verified that boundens yeast and bacillus B10 stimulated chicken bone marrow dendritic cells, increasing TLR 1, TLR 2, TLR 4, and TLR 15 expression (Rajput *et al*., 2014).

Bacillus coagulans not only has the characteristics of lactobacilli and bifidobacteria, but is also resistant to acid environments and high temperatures (Hyronimus *et al.*, 2000; Ripamonti *et al.*, 2009). Previous experimental studies showed that *Bacillus coagulans* was able to regulate cell factors, enhanced the phagocytosis of phagocytic cells, enhanced the activity of NK, T and B cells, and increased the expression of IgA, IgG and IgM (Kodali *et al.,* 2008). Consistently, the results of the present experiment showed that *Bacillus coagulans* can enhance the immune function of the gut of yellow-feathered broilers.

ACKNOWLEDGMENTS

This work was supported by a program from China Fujian laying hen industry system program (NO. K83139297 (2013-2017)).

REFERENCES

Anderson RC, Cookson AL, McNabb WC, Park Z, Mccann MJ. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiology* 2010; 10: 316.

Apajalahti J, Kettunen A, Graham H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World Poultry Science Journal* 2004;60 (2): 223-232.

Jindal A, Kocherginskaya S, Mehboob A, Robert M,Mackie RI,Zillles LR. Antimicrobial use and resistance in swine waste treatment systems. *Applied and Environmental Microbiology* 2006;72 (12): 7813-7820.

Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews Immunology* 2008;8 (6): 411-420.

Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. Restoration of barrier function in injured intestinal mucosa. *Physiological Reviews* 2007;87(2): 545–564.

Caspary WF. Physiology and pathophysiology of intestinal absorption. *American Journal of Clinical Nutrition* 1992; (55): 299-308.

Castillo NA, Perdigón G, Leblanc A de M. Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella* enterica serovar typhimurium infection in mice. *BMC Microbiology* 2011; 11: 177-189.

Cui YL, Run SC, Wan FC. Bacteriostasis of Bacillus coagulans TBC 169 to enteropathogenic bacteria. *Chinese Journal of Microecology* 2005; 17 (5): 333-338.

Dai C, Zhao D H, Jiang M. VSL3 probiotics regulate the intestinal epithelial barrier *in vivo* and *in vitro* via the p38 and ERK signaling pathways. *International Journal of Molecular Medicine* 2012; 29 (2): 202-208.

Duerr CU, Hornef MW. The mammalian intestinal epithelium as integral player in the establishment and maintenance of host-microbial homeostasis. *Seminars Immunology* 2012; 24 (1): 25-35.

Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'Neil DA. Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005; 54(2): 242-249.

Gómez-Llorente C, Muñoz S, Gil A. Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. *Proceeding of Nutrition Society* 2010; 69(3): 381-389.

Giang HH, Viet T Q, Linberg J E, Linberg JE. Groeth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livestock Science* 2010;129(1-3): 95-103.

Honda H, Gibson GR, Farmer S, Keller D, McCartney AL. Use of a continuous culture fermentation system to investigate the effect of GanedenBC30 (Bacillus coagulans GBI-30, 6086) supplementation on pathogen survival in the human gut microbiota. *Anaerobe* 2011; 17 (1): 36-42.

Hyronimus B, Le Marrec C, Sassi AH, Deschamps A. Acid and bile tolerance of spore-forming lactic acid bacteria.*International Journal of Food Microbiology* 2000;61(2-3):193-197.

Isolauri E, Sutas Y, Kankaanpaa P,Arvilommi H,Salminen S. Probiotics: effects on immunity. *American Journal of Clinical Nutrition* 2001; 72 (2): 444-450.

Jin Er-hui, Chen Yao-xing,Wang Qun,Qiao En-mei,Wu Guo-zhong,Li Sheng-he. Effect of the bacillus probiotics on blood cells anf structure of immune organs in broilers. Acta veterinaria et zootechnica sinica2013;44(5):778-787.

Kailova L, Mount Patrick SK, Arganbright KM, Halpem MD, Kinouchi T,Dvorak B. *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. *American Journal of Physiology Gastrointestinal Liver Physiology* 2010; 299(5):1118-1127.

Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* 2010;11: 373-384.

Kodali VP, Sen R. Antioxidant and free radical scavenging activities of an exopolysaccharide from a probiotic bacterium. *Journal of Biotechnology* 2008;3(2): 245-251.

Sahoo KC, Tamhankar AJ, Johansson E, Lundborg CS. Antibiotic use, resistance development and environmental factors: a qualitative study among healthcare professionals in Orissa, India. *BMC Public Health* 2010; 10(1):1-10.

Lebeer S, Vanderleyden J, De Keersmaecker CJ. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nature Review Microbiology* 2010; 8(3): 171-184.

Lei XJ, Piao X , Ru Y, Zhang H, Peron A, Zhang H. Effect of bacillus amyloliquefaciens-based direct-fed microbial on performance, nutrient utilization, intestinal morphology and cacal microflora in broiler chickens. *Asian-Australasian Journal of Animal Science* 2015;28(2):239-246.

Lin LH, Ke FR, Zhan TT, Xu LH, Wang QX, Wang CK, Huang SW. Effects of Bacillus coagulans on Performance, Serum Biochemical Indices and Antioxidant Function of Yellow Broilers. *Chinese Journal of Animal Nutrition.* 2014, 26(12): 2806-2813.

Liu Y, Fatheree NY, Mangalat N, Rhoads JM. *Lactobacillus reuteri* strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF- κB signaling in the intestine. *American Journal of Physiology Gastrointestinal Liver Physiology* 2012; 302(6): 608-617.

Ma K, Maeda T, You H, Shirai Y. Open fermentative production of L-lactic acid with high optical purity by thermophilic *Bacillus coagulans* using excess sludge as nutrient. *Bioresource Technology* 2014;151: 28-35.

Medici M, Vinderola C, Perdigon G. Gut mucosal immunomodulation by probiotic fresh chess. International Dairy Journal 2004,14(7):611-618.

Miriam BB, Julio PD, Sergio MQ, Carolina G L, Angel G. Probiotic Mechanisms of Action. *Annal of Nutrition Metabolism* 2012;61:160–174.

Moore PR, Evension A, Luckey TD, Mccoy E, Elvehjem CA, Hart EB. Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick.*The Journal of Biological Chemistry* 1946;165: 437-441.

Natsir MH, Sjofjan O, Umam K, Manab A, Widodo E. Effects of liquid and encapsulated lactic acid in broiler diets on performances, intestinal characteristics and intestinal microflora.*The Journal of Poultry Science* 2010; 47: 240-243.

Pan X, Qiang Z, Ben W, Chen M. Residual veterinary antibiotics in swine manure from concentrated animal feeding operations in Shandong Province, China. *Chemosphere* 2011; 84 (5): 695-700.

Rajput IR, Li LY, Xin X, Wu BB, Juan ZL, Cui DY, Li WF. Effect of *Saccharomyces boulardii* and *Bacillus subtills* B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. *Poultry Science* 2013;92 (4): 956-965.

Rajput IR, Hussain A, Li YL, Zhang X, Xu X, Long MY, You DY, Li WF. *Saccharomyces boulardii* and *Bacillus subtilis* B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs. *Journal of Cell Biochemistry* 2014; 115 (1): 189-198.

Riazi S, Wirawan RE, Badmaev V, Chikindas ML. Characterization of lactosporin, a novel antimicrobial protein produced by *Bacillus coagulans* ATCC 7050. *Journal of Applied Microbiology* 2009; 106(4): 1370-1377.

Ripamonti B, Agazzi A, Baldi A, Balzaretti C, Bersani C, Pirani S, Rebucci R, Savoini G, Stilla S, Stenico A, Domeneghini C. Administration of Bacillus coagulans in calves:recovery from faecal samples and evaluation of functional aspects of spores. *Veterninary Research Communication* 2009; 33(8):991-1001.

Durbin RK, Kotenko SV, Durbin JE.Interferon induction and function at the mucosal surface. Immunological reviews 2013;255(1): 25-39.

Sheil B, Shanahan F, Omahony L. Probiotic effects on inflammatory bowel disease. *The Journal of Nutrition* 2007; 137 (3): 819S-824S.

Smither R, Lott AF, Dalziel RW, Ostler DC. Antibiotic residues in meat in the United Kingdom; an assessment of specific tests to detect and identify antibiotic residues. *Journal of Hygiene, Camb* 1980; 85(3):359- 359.

Vahjen W, Osswald T, Schäfer K, Simon O. Comparison of a xylanase and a complex of non starch polysaccharide-degrading enzymes with regard to performance and bacterial metabolism in weaned piglets. *Archives of Animal Nutrition* 2007; 61(2):90-102.

Wang N, Wang G, Hao JX, Ma JJ, Wang Y, Jiang XY, Jiang HQ.Curcumin ameliorates hydrogen peroxide-induced epithelial barrier disruption by upregulating heme oxygenase-1 expression in human intestinal epithelial cells.*Digestive Diseases and Science* 2012;57(7):1792-1801.

Wells JM. Immunomodulatory mechanisms of lactabacilli. *Microbial Cell Factories* 2011; 10 (suppl 1): S17.

Wells JM, Rossi O, Meijerink M, Baarlen P. Epithelial crosstalk at the microbiota-mucosal interface. *Proceedings of the National Academy Sciences* 2011;108 (1): 4607-4614.

Zhang L, Xu Y Q, Liu H Y, Lai T,Ma JJ, Wang JF,Zhu YH.Evaluation of *Lactobacillus rhamnosus* GG using an Escherichia coli K88 model of piglet diarrhoea: Effects on diarrhoea incidence, faecal microflora and immune responses. *Vet Microbiol* 2010; 141 (1-2): 141-148.

Zheng Z, Ma C, Gao C,Li F,Qin J, Zhang H,Wang K,Xu P. Efficient conversion of phenylpyruvic acid to phenyllactic acid by using whole cells of Bacillus coagulans SDM. *PLoS One* 2011;6 (4): e19030.