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Effect of Bacillus subtilis natto on Growth Performance in Muscovy Ducks

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ABSTRACT

The aim of the present study was to determine whether dietary *Bacillus subtilis natto* could affect growth performance of Muscovy ducks. A total of 120 hundred Muscovy ducks at the age of 1 day were randomly assigned to four groups (30 Muscovy ducks/group), and fed with diets supplemented with 0% (control group), 0.1%, 0.2%, and 0.4% *Bacillus subtilis natto*, respectively during the 6-week feeding period. Weight gain, feed intake and feed conversion efficiency of Muscovy ducks were significantly improved by the dietary addition of *Bacillus subtilis natto*, and the results were more significant in 0.4% dietary *Bacillus subtilis natto* treatment group; Also, *Bacillus subtilis natto* reduced *Escherichia coli and Salmonella* colonies, and increased lactobacilli population in the ileum and the cecum. Biochemical parameters, including total protein, GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvic transaminase), AKP (alkaline phosphatase), triiodothyronine (T_3) and tetraiodothyronine (T_4) contents ($p<0.05$) in the serum of Muscovy ducks were significantly improved ($p<0.01$), while urea nitrogen, glucose, triglyceride, total cholesterol concentrations decreased when *Bacillus subtilis natto* was added to the diets ($p<0.05$), and improved duodenum and immune functions. However, the results above were not significantly different between birds fed 0.1% *Bacillus subtilis natto* supplemented diets and the control group ($p>0.05$). The results of the present study indicate that diets with 0.4% *Bacillus subtilis natto* improved the growth performance of Muscovy ducks by increasing the absorption of protein, simulating hormone secretion, suppressing harmful microflora, and improving the duodenal structure and immune functions of Muscovy ducks. It is suggested that *Bacillus subtilis natto* is a potential candidate to be used use as a probiotic to improve the growth performance of Muscovy ducks.

INTRODUCTION

Probiotics have been used as feed additives to replace antibiotics, as these may induce resistance and leave residues in animal products (Steiner, 2006). The main probiotic strains are *Bacillus*, *Lactobacillus*, *Streptococcus*, *Saccharomyces* and *Aspergillus* (Tannock, 2001). *Bacillus subtilis* may secrete protease, amylase, and lipase, which are important to improve the growth performance and to control diseases in animals (Santoso *et al*., 2001). Therefore, development and application of *Bacillus subtilis* as a feed additive may be useful.

Bacillus subtilis natto, a strain of *Bacillus subtilis*, is the basis for the production of the traditional Japanese food "natto", made from soybeans. Soybean natto, fermented by *Bacillus subtilis natto*, may potentially become a functional and health food because of its high antioxidant activity (Hu *et al*., 2010). Furthermore, previous findings demonstrated that many enzymes purified from *Bacillus*

subtilis natto , such as methyltransferase, gamma-glutamyltranspeptidase, alanine dehydrogenase, leucine dehydrogenase, and serine proteinase, are functional (Shih *et al*., 2005). The protein derived from *Bacillus subtilis natto* had both fibrinolytic and antithrombotic effects similar to heparin (Omura *et al*., 2005), enhancing endogenous thrombolysis in a dose-dependent manner (Yamashita *et al*., 2003). A lipopeptide biosurfactant purified from *Bacillus subtilis natto* was able to inhibit the proliferation of human breast-cancer cells (Cao *et al*., 2009) and human leukemia cells (Wang *et al*., 2007), indicating the antitumour activity of *Bacillus subtilis natto*. Also, it was shown that *Bacillus subtilis natto* contains nattokinase, which displays strong fibrinolytic activity and activates other fibrinolytic enzymes, such as pro-urokinase and tissue plasminogen activator (Yanagisawa *et al*., 2010).

There are few studies reported in literature on the effects of *Bacillus subtilis natto* supplementation in the diets of fish, livestock and poultry (Samanya and Yamauchi, 2002; Chen *et al*., 2009; Liu *et al*., 2009; Sun *et al*., 2010) because of its many diverse functions. To date, no study was reported on the supplementation *Bacillus subtilis natto* in the diets of Muscovy ducks. The present study evaluated the effects of *Bacillus subtilis natto* on the growth performance of Muscovy ducks.

MATERIALS AND METHODS

Experimental Design and Treatments

A total of 120 Muscovy ducks (*Cairina moschata*) with almost the same body weight (45-48g) were obtained from a commercial breeding company (Donglian, Shaoguan, China), and randomly distributed into four groups, with 30 Muscovy ducks per group. Birds were daily fed a commercial diet and water *ad libitum* during the 6-week study. Ingredients and nutritional composition of the basal diet, formulated were according with INRA requirements (1984), are shown in Table 1. All data were measured, except for metabolic energy (ME).

Diets were supplemented with four levels of *Bacillus subtilis natto*: diets with 0% of *Bacillus subtilis natto* was set as control group, and those with 0.1%, 0.2%, and 0.4% *Bacillus subtilis natto* were set as treatment groups. Diets were supplied daily, and clean drinking water was supplied *ad libitum* during the 6-week feeding period. Birds were exposed to infrared lamp before 3 weeks and then to incandescent light a period of 12-hour (12L:12D) each day. Temperature and ventilation were controlled. The temperature was set at 20-27°C throughout the experiment.

Table 1 – Ingredients and nutritional compositios of the experimental basal diets %

Basal diets	Bird age	
	1-3 weeks	3-6 weeks
Ingredients (%)		
Ground yellow corn	60.00	24.70
Rough rice	---	40.00
Wheat bran	7.00	10.00
Soybean meal	21.25	13.55
Fish meal	5.00	3.50
Yeast Powder	4.00	3.00
Soybean oil	---	2.00
Calcium monohydrogen phosphate	1.00	1.50
Salt	0.25	0.25
Mineral and vitamin premix[1]	1.50	1.50
Analyzed chemical composition		
Crude protein (%)	19.20	16.00
Calcium (%)	1.16	1.25
Available phosphorus (%)	0.55	0.65
Lysine (%)	1.00	0.65
Methionine (%)	0.50	0.33
Methionine + Cystine (%)	0.85	0.63
ME[2] (MJ/kg)	11.50	9.80

[1] Supplied per kilogram of premix: vitamin A, 1,200,000 IU; vitamin E, 2000 IU; vitamin D_3, 250,000 IU; vitamin K_3, 200 mg; vitamin B_1, 100mg; vitamin B_2, 800 mg; vitamin B_6, 200mg; vitamin B_{12}, 1.0 mg; folic acid, 200 mg; biotin, 5 mg; niacin, 7,500mg; pantothenic acid,1000 mg; Mn, 10.0 g; Zn, 10.0 g; Fe, 7.5 g; Cu, 0.5 g; I, 200 mg; Cr, 10.0 mg; and Se, 15 mg.

[2] The data is calculated

Performance Calculation and Sample Collection

Ducks were weighed with one day of age and at the end of the experiment. Daily feed intake was recorded daily. Moreover, feed conversion ratio (feed intake/ weight gain) was calculated for during the 6-week feeding period.

Ten ducks per group were selected and weighed on day 42 of the feeding trial. Blood samples (8.0 ml) from each individual were collected from the heart with sterilized syringes and needles. Then the ducks were killed by exsanguination and were dissected. The thymus, spleen and bursa of Fabricius were collected and weighed. The immune organ index (in g/kg) was calculated by dividing immune organ weight by the body weight of the ducks. Furthermore, segments of approximately 2cm were collected from duodenum (posterior to gizzard) for intestinal morphometry. Also, 2cm of the ileum and the cecum with contents were stored in sterile plastic bags to measure intestinal tract microflora population.

Analysis of serum biochemical parameters

The collected blood samples were centrifuged (4000 rpm) for 6min and then the obtained serum samples were stored at -70°C until further analysis. The concentrations of total protein, glucose, triglyceride, total cholesterol, urea nitrogen, GOT, GPT and AKP were measured using enzyme reagent methods. The assay kits were provided by Ningbo Cicheng Biochemistry Reagent Factory.

The levels of T_3 and T_4 in serum were analyzed using the method of radio-immunity (RI). The assay kits of hormones were provided by Beijing North Institute of Biological Technology (20a Panjiamiao, Fengtai District, Beijing, China).

Intestinal Morphometry

According to the method described by Becak and Paulete (1976), the duodenum segments collected on the last day of the feeding period were placed on polystyrene sheets, longitudinally opened, washed in saline solution, fixed in Bouin's solution for 24h, and processed until paraffin embedding. Each fragment was submitted to semi-serial cuts (5mm thick) and stained using the hematoxylin-eosin method.

In the morphometric study of duodenum, images were captured using a light microscope and a system that analyzes computerized images. The height and width of 30 villi, the depth of 30 crypts, and the thickness of 30 muscular layers were measured in all segments per replicate. The villus perimeter was based on the villus circumference and the distance between neighboring villi. Crypt depth was the shortest vertical distance from the villus contact point to the mucous membrane. The muscle layer was the shortest vertical distance from the point between the epimysium and annular muscle to the mucous membrane.

Analysis of intestinal Tract Microflora Population

Ileum and cecum contents were diluted 10-fold with buffered peptone water, vortexed for 2 min and the supernatant was preserved at -70°C. One hundred microliters of supernatant was smeared onto MacConkey agar and incubated at 37°C for 24 h to enumerate *Escherichia coli* colonies, on Rogosa agar at 37°C for 48 h to measure lactobacilli population, or on SS agar at 37°C for 24 h to count *Salmonella* colonies, respectively, in triplicate.

STATISTICAL ANALYSIS

The experiment was conducted according to a completely randomized design. The SAS (Statistical Analysis System, SAS Institute Inc, Cary, NC, USA, Version 9.1.3) software package was used for all statistical analysis. Duncan's multiple range test ($p<0.05$) was used to test the significance of the difference among means. All data were expressed as means ± SD. Differences were considered significant at the level of $p<0.05$.

RESULTS

Effects of *Bacillus subtilis natto* on growth performance of Muscovy ducks

The effect of *Bacillus subtilis natto* on the growth performance in Muscovy ducks fed with the diets supplemented with 0%, 0.1%, 0.2%, and 0.4 % *Bacillus subtilis natto* is shown in Table 2. After a feeding period of 6 weeks, weight gain ($p<0.05$), feed intake ($p<0.05$) and feed conversion ratio ($p<0.01$) of Muscovy ducks significantly improved when 0.2% and 0.4% *Bacillus subtilis natto* were added to the diets, and the results were more evident in the 0.4% dietary *Bacillus subtilis natto* treatment; however, weight gain, feed intake and feed conversion ratio were not significantly different between Muscovy ducks fed with 0.1% *Bacillus subtilis natto* supplemented diets compared with the control group ($p>0.05$).

Table 2 – Effect of the dietary addition of *Bacillus subtilis natto* on the growth performance of Muscovy ducks[1]

Items	Levels of *Bacillus subtilis natto* (%)				SEM
	0 (control)	0.1	0.2	0.4	
Weight gain (g/bird/d)	32.38^{a}	32.47^{a}	33.47^{b}	34.49^{c}	0.19
Feed intake (g/bird/d)	88.11^{a}	87.67^{a}	88.22^{ab}	89.08^{b}	0.17
Feed conversion[2] (g/g)	2.72^{a}	2.70^{a}	2.64^{b}	2.58^{c}	0.07

^{a,b,c} Different letters for the same line denote significant differences ($p< 0.05$).

[1] Values represent the means ± SEM of 10 Muscovy ducks per group.

[2] Feed conversion = feed intake: weight gain (g:g).

Effects of *Bacillus subtilis natto* on serum biochemical parameters of Muscovy ducks

The levels of total protein, glucose, triglyceride, total cholesterol, urea nitrogen, GOT, GPT, AKP, T_3 and T_4 in the different treatments were measured and are shown in Table 3. The results indicated that

Table 3 – Effect of dietary *Bacillus subtilis natto* levels on biochemical parameters of Muscovy ducks[1]

Items	Levels of *Bacillus subtilis natto*, %				SEM
	0 (control)	0.1	0.2	0.4	
Total protein (g/l)	38.79[a]	38.62[a]	42.53[b]	46.43[c]	0.62
Glucose (mg/ml)	0.10[a]	0.094[ab]	0.092[b]	0.088[b]	0.01
Triglyceride (mg/ml)	16.35[a]	16.15[ab]	15.84[b]	14.35[c]	0.14
Total cholesterol (mg/ml)	2.07[a]	2.02[a]	1.85[b]	1.76[b]	0.03
Urea nitrogen (mg/ml)	0.107[a]	0.099[a]	0.087[b]	0.077[b]	0.003
GOT(U/l)	56.74[a]	59.19[b]	60.11[b]	64.52[c]	0.48
GPT(U/l)	65.49[a]	68.42[b]	72.43[c]	76.65[d]	0.70
AKP(U/l)	134.58[a]	136.84[b]	143.83[c]	158.55[d]	1.50
T_3 (ng/ml)	5.28[a]	5.71[b]	6.64[c]	7.29[d]	0.13
T_4 (ng/ml)	15.05[a]	16.11[b]	17.36[c]	18.63[d]	0.22

[a,b,c,d] Different letters in the same row indicate significant differences ($p< 0.05$).

[1] Values represent the means ± SEM of 10 Muscovy ducks per group.

treatment with 0.1% dietary *Bacillus subtilis natto* had little effect on total protein, glucose, triglyceride, total cholesterol and urea nitrogen levels ($p>0.05$), but increased the secretion of GOT, GPT, AKP, T_3 and T_4 ($p<0.05$). Total protein, GOT, GPT, AKP, T_3 and T_4 blood levels significantly increased ($p<0.05$), and glucose, triglyceride, total cholesterol and urea nitrogen levels significantly decreased ($p<0.01$) when the birds were fed with 0.2% and 0.4% *Bacillus subtilis natto* in diets.

Effects of *Bacillus subtilis natto* on microflora population in ileum and cecum of Muscovy ducks

As shown in Table 4, *Escherichia coli*, *Salmonella*, and *lactobacilli* levels changed when *Bacillus subtilis natto* was added to the diets. Compared with the control treatment, 0.1% *Bacillus subtilis natto* reduced *Escherichia coli* and *Salmonella* counts, and increased *lactobacilli counts*, but this effect was not significant ($p>0.05$); 0.2% *Bacillus subtilis natto* promoted better effects ($p<0.05$). Furthermore, the effect of 0.4% *Bacillus subtilis natto* on the microflora population was more significant ($p<0.01$).

Effects of *Bacillus subtilis natto* on development of duodenum of Muscovy ducks

The intestinal morphology of duodenum in different treatments is shown in Table 5. Compared with the control group, birds fed 0.1% *Bacillus subtilis natto* had a higher villus height and villus width in the duodenum ($p<0.05$), but there was no statistically significant difference in crypt depth and muscular layer thickness ($p>0.05$). The dietary levels of 0.2% and 0.4% *Bacillus subtilis natto* clearly improved the growth of duodenum and promoted the longest villus height and largest absorptive surface area. Villus width, villus height, crypt depth, and muscular layer thickness results demonstrated that 0.1% and 0.2% *Bacillus subtilis natto* were able to promote the development of duodenum, but failed to match the performance of 0.4% *Bacillus subtilis natto* ($p<0.01$).

Table 4 – Effect of different levels *Bacillus subtilis natto* on microflora in Muscovy ducks[1]

Items		Levels of *Bacillus subtilis natto*, %				SEM
		0 (control)	0.1	0.2	0.4	
Ileum	*Escherichia coli*	6.88[a]	6.66[ab]	6.45[b]	5.98[c]	0.07
	Salmonella	6.19[a]	5.68[b]	5.33[c]	5.18[c]	0.07
	Lactobacilli	7.66[a]	7.71[a]	7.95[b]	8.31[c]	0.05
Cecum	*Escherichia coli*	7.91[a]	7.79[a]	7.15[b]	6.91[b]	0.86
	Salmonella	6.71[a]	6.54[ab]	6.37[b]	5.95[c]	0.60
	Lactobacilli	8.32[a]	8.13[a]	8.61[ab]	9.23[c]	0.89

[a,b,c] Different letters in the same row indicate significant differences ($p< 0.05$).

[1] values represent the means ± SEM of 10 Muscovy ducks per group.

Table 5 – Effect of *Bacillus subtilis natto* on duodenum morphology of Muscovy ducks[1]

Items	Levels of *Bacillus subtilis natto*, %				SEM
	0 (control)	0.1	0.2	0.4	
Villus width (μm)	132[a]	138[b]	140[b]	146[c]	0.89
Villus height (μm)	1323[a]	1345[b]	1425[c]	1478[d]	10.09
Crypt depth (μm)	250[a]	247[ab]	242[c]	230[d]	1.58
Muscular layer thickness (μm)	214[a]	218[ab]	232[c]	247[d]	2.19

[a,b,c,d] Different letters in the same row indicate significant differences ($p< 0.05$).

[1] The height and width of 30 villi, the depth of 30 crypts and the thickness of 30 muscular layers were measured per replicate.

Effect of *Bacillus subtilis natto* on immune functions in mucovy ducks

Bacillus subtilis natto supplementation tended to improve the immune functions of Muscovy ducks. The results in Table 6 show that, compared with the control group, the indices of thymus, spleen and bursa of Fabricius improved when *Bacillus subtilis natto* was added to the diets, indicating *Bacillus subtilis natto* may improve the immune functions in Muscovy ducks. The addition of 0.1% did not influence the immune function ($p>0.05$), while 0.2% and 0.4% dietary addition of *Bacillus subtilis natto* promoted significant results ($p<0.01$).

Table 6 – Effect of *Bacillus subtilis natto* on immune organ indices in Muscovy ducks[1], g/kg

Items	Levels of *Bacillus subtilis natto*, %				SEM
	0 (control)	0.1	0.2	0.4	
Thymus	2.18[a]	2.20[ab]	2.54[c]	2.85[d]	0.05
Spleen	0.73[a]	0.79[ab]	0.83[bc]	0.86[c]	0.01
Bursa of Fabricius	1.26[a]	1.29[ab]	1.37[c]	1.42[d]	0.02

[a,b,c,d] Different letters in the same row indicate significant differences ($p< 0.05$).

[1] values represent the means ± SEM of 10 Muscovy ducks per group.

DISCUSSION

Previous studies showed that probiotics used as feed additives to replace antibiotics tended to improve the animal growth and health (Steiner *et al*., 2006). Zhang *et al*. (2005) reported that diets supplemented with 0.3% yeast (*S. cerevisiae*) cell components improved weight gain, feed intake, and feed conversion ratio of Ross broilers. Diets supplemented with 0.1% *B. subtilis* powder increased the weight gain of 10-d-old male Arbor Acres broiler chicks maintained in a high temperature and humidity environment (Jin *et al*., 1996). Santoso *et al*. (1995, 2001) also demonstrated that diets supplemented with 0.5% *B. subtilis* fermentation product improved broiler growth performance. Diets supplemented with *Bacillus licheniformis* and *B. subtilis* improved the feed conversion ratio of AvianxAvian broilers (Mutus *et al*., 2006).

The findings obtained with *Bacillus subtilis natto* suggest that it may improve the growth performance of animals and as well as profitability. For instance, milk supplemented with *Bacillus subtilis natto* fed directly to calves promoted better growth performance by improving the average daily gain and feed conversion ratio, allowed earlier weaning, and benefitted the immune function of calves (Sun *et al*., 2010). *Bacillus subtilis* isolated from natto improved shrimp growth performance by increasing feed digestibility (Liu *et al*., 2009). When dietary *Bacillus subtilis natto* was fed chickens for 28 days, birds tended to present better growth performance and intestinal histology, as well lower ammonia emmission. *Bacillus subtilis* has the potential to be a beneficial microorganism in chickens (Samanya *et al*., 2002). In this study, we found that low levels of *Bacillus subtilis natto* in the diets of Muscovy ducks did not influence growth performance compared with the control group; however, the supplementation of 0.2% and 0.4% *Bacillus subtilis natto* to the diets of Muscovy ducks improved their weight gain, feed intake, and feed conversion ratio.

Our findings further indicate that the dietary supplementation of *Bacillus subtilis natto* significantly increased total protein, GOT, GPT, AKP, T_3 and T_4 contents in serum of Muscovy ducks, while decreased urea nitrogen, glucose, triglyceride, total cholesterol levels. Malmolf (1988) reported that the serum urea nitrogen concentration was related with the status of protein metabolism and amino acid balance. The serum concentration of urea nitrogen decreases when amino acids are well balanced. Total serum protein concentration is a parameter that reflects the ability of the liver to synthesize protein. This study indicated that *Bacillus subtilis natto* tended to increase the concentration of serum urea nitrogen and total serum protein. This may be related to enhanced protein intake and balanced amino acid status when *Bacillus subtilis natto* is added to the diet of Muscovy ducks.

Serum GOT and GPT are critical parameters to measure liver function. This experiment showed that *Bacillus subtilis natto* increased the serum GOT and

GPT concentration. Alkaline phosphatase (AKP) is an enzyme that can catalyzes many kinds of lecithoid compounds to release inorganic phosphorus, which is extensively present in animal tissues, exerting its important function in the formation of the skeleton and fat. Results showed that the activity of AKP was high in *Bacillus subtilis natto* treated groups, but its mode of action needs to be further studied.

The levels of T_3 and T_4 in the peripheral blood plays several physiological functions, such as facilitating the differentiation, growth and development of tissues, stimulating DNA transcription and mRNA formation, promoting the synthesis of protein and enzymes, increasing the utilization of carbohydrates, and enhancing the disintegration of starch and fat (Ooi *et al*., 2004). After feeding *Bacillus subtilis natto* for six weeks, the levels of T_3 and T_4 in the blood of Muscovy ducks were measured and the results indicated that *Bacillus subtilis natto* increased the concentrations of T_3 and T_4, thus promoting enhanced nutrient metabolism and absorption and performance of Muscovy ducks. The changes in duodenum structure observed in this study are also better for absorption in nutrients.

Bacillus subtilis natto changed the microflora population in the intestine. It enhanced the growth and viability of lactobacilli through production of catalase and subtilisin, demonstrating the potential usefulness of *Bacillus subtilis natto* as a probiotic (Hosoi *et al*., 2000). Subtilisin, produced by *Bacillus subtilis* was found to be effective against staphylococcal enterotoxins (Osawa and Matsumoto, 1997). The administration of intact spores of *Bacillus subtilis natto* did not affect fecal *Enterobacteriaceae* and *Enterococcus spp.*, but affected fecal *Bacteroidaceae* and *Lactobacillus spp*.; however, these changes require monosaccharides or oligosaccharides (Hosoi *et al*., 1999). In our study, it was observed that *Bacillus subtilis natto* provided better intestinal environment by reducing *Escherichia coli* and *Salmonella* numbers and increasing lactobacilli population in ileum and cecum of Muscovy ducks.

Thymus, spleen and bursa of Fabricius are the main immune organs involved in humoral immunity and cell immunity of animals. Thymus is the site of T cell maturation, and the bursa of Fabricius is required for B cell (part of the immune system) development in birds. The function of the spleen and its responses to antigens are much like those of lymph nodes (Cooper *et al*., 1966). The dietary addition of *Bacillus subtilis natto* increased the percentage of T and B lymphocytes in the spleen, suggesting that dietary *Bacillus subtilis natto* had an effect on cellular immune responses (Inooka *et al*., 1986). Chickens fed *Bacillus subtilis natto* in the diet from hatching until 15 or 30 days of age showed an increase in the ability to produce hemagglutinating antibody titers (Inooka and Kimura, 1983). Our results showed that *Bacillus subtilis natto* increased thymus, spleen and bursa of Fabricius indices, indicating *Bacillus subtilis natto* could enhance immune functions of Muscovy ducks.

In conclusion, the results of this study showed that the supplementation of *Bacillus subtilis natto* to the diets of Muscovy ducks improves their growth performance by increasing the absorption of protein, promoting hormone secretion, suppressing harmful microflora, promoting better development of the duodenum structure and enhancing the immune functions of Muscovy ducks. In addition, the results suggest that *Bacillus subtilis natto* is a potential candidate to be used as a probiotic to improve the growth performance of Muscovy ducks.

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