

Effects of dietary supplementation of probiotics (*Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto*) on broiler muscle development and meat quality

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Received: 23.06.2014 • Accepted: 19.09.2014 • Published Online: 01.04.2015 • Printed: 30.04.2015

Abstract: This paper aims to evaluate the effects of probiotics (*Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto*) on muscle development and meat quality of broilers. In this study, 480 broilers (1 day old) were randomly divided into 4 groups. Group A was fed with a basal diet. The basal diets of Group B, Group C, and Group D were respectively supplemented with 50 mg/kg chlortetracycline, 200 mg/kg probiotics, and 400 mg/kg probiotics. On day 21 and day 42, in each group, 18 randomly selected broilers were tested to determine muscle performances. On day 21, compared with Group A, Group C and Group D showed no significant difference in muscle weight, muscle fiber diameter, meat color, drip loss, shear loss, or cooking loss ($P > 0.05$). On day 42, compared with Group A, Group C and Group D showed significant increases in muscle weight and muscle fiber diameter and significant decreases in b^* value, drip loss, cooking loss, and shear force ($P < 0.05$). Compared with Group A and Group B, Group C and Group D showed obviously improved muscle microstructure. In conclusion, diets supplemented with 200 or 400 mg/kg probiotics could promote muscular development and meat quality of broilers in the late stage of growth.

Key words: Probiotics, broiler, muscle development, meat quality

1. Introduction

Probiotics are green feed additives without pollution or residue, and they will not lead to drug resistance (1,2). Probiotics can promote animal growth, increase feed conversion ratio, enhance immune function, improve the intestinal microflora, and affect lipid metabolism and other functions. Dietary supplementation of probiotics can significantly increase average daily gain and feed conversion ratio of broilers, pigs, and goats (3–5); the quantity of intestinal lactic acid bacteria and *Bacillus cereus* of hens (6); the weights and indexes of the spleen and bursa as well as serum IgG levels of broilers (7,8); and intestinal villus height, epithelial cell area, and number of mitotic cells of broilers (9). Kalavathy et al. (10) found that probiotics could reduce serum total cholesterol level, low-density lipoprotein level, and abdominal fat deposition. *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto* are commonly used probiotic preparations. Dietary supplementation of single-strain probiotics can significantly improve animal production performance, regulate intestinal microflora imbalance, and enhance immune functions (11). However, dietary supplementation of multistrain probiotics is seldom reported. In this paper,

with broilers as experimental animals, we study the effects of dietary supplementation of multistrain probiotics (*Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto*) on muscle development and meat quality. This study can provide a scientific basis for the application of probiotics in poultry feeding.

2. Materials and methods

2.1. Experimental animals and drugs

In total, 480 Arbor Acres (AA) broilers (male or female) of 1 day old were purchased from the Animal Husbandry Park of Anhui Science and Technology University. The probiotics used in the experiment were white powders of multistrain probiotics with the content of 1×10^{10} cfu/g. Probiotics containing *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto* were purchased from Jiaying Kerui Biotechnology Co., Ltd. (Jinhua, China).

2.2. Diet preparation

According to the basal diet of broiler chicks recommended by the US National Research Council, a corn-soybean basal diet was prepared. The compositions and nutrient levels of the basal diet are provided in Table 1.

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Table 1. Nutrient compositions of the basal diet.

Ingredients (%)	Days 1–21	Days 22–42	Nutrition levels	Days 1–21	Days 22–42
Corn	58.00	62.00	Crude protein (%)	21.46	19.08
Soybean meal	32.00	27.50	Lysine (%)	1.21	1.04
Fish meal	3.00	2.00	Methionine (%)	0.35	0.31
Soybean oil	2.00	3.50	Calcium (%)	1.10	1.02
Vitamin mineral premix ¹	5.00	5.00	Metabolic energy (kcal/kg)	2919	3032
			Phosphorus (%)	0.59	0.44

¹Vitamin mineral premix contained the following per kilogram of diet on days 1–21: vitamin A, 24,000 IU; vitamin D₃, 5400 IU; vitamin E, 560 IU; vitamin K₃, 56 mg; vitamin B₁, 16 mg; vitamin B₂, 108 mg; vitamin B₆, 18 mg; vitamin B₁₂, 0.25 mg; nicotinic acid, 650 mg; pantothenic acid, 240 mg; folic acid, 12 mg; choline chloride, 10 g; copper, 100 mg; iron, 400 mg; zinc, 960 mg; manganese, 960 mg; iodine, 10 mg; selenium, 10 mg; calcium, 14%; total phosphorus, 2.5%; salt, 4%; moisture, 12%.

Vitamin mineral premix contained the following per kilogram of diet on days 22–42 days: vitamin A, 20,000 IU; vitamin D₃, 5000 IU; vitamin E, 480 IU; vitamin K₃, 50 mg; vitamin B₁, 16 mg; vitamin B₂, 90 mg; vitamin B₆, 18 mg; vitamin B₁₂, 0.12 mg; nicotinic acid, 600 mg; pantothenic acid, 240 mg; folic acid, 8 mg; choline chloride, 10 g; copper, 200 mg; iron, 400 mg; zinc, 1200 mg; manganese, 960 mg; iodine, 10 mg; selenium, 20 mg; calcium, 18%; total phosphorus, 2.3%; salt, 8%; moisture, 12%.

2.3. Animal grouping and feeding

First, according to the grouping principle of similar weight, 480 AA broiler chicks (1 day old) were randomly divided into 4 experimental groups (A, B, C, and D) with 120 chicks in each group having 6 replicates. The dimensions of the feeding space were 2.0 m × 1.0 m × 1.0 m. The experimental period was 42 days. The control group (Group A) was fed with the basal diet (Diet A). The antibiotic group (Group B) was fed with the basal diet supplemented with 50 mg/kg chlortetracycline (Diet B), which was provided by Kangdi Feed Co., Ltd. (Hefei, China). For the 2 probiotic groups (Group C and Group D), the basal diets were respectively supplemented with 200 mg/kg probiotics (Diet C) and 400 mg/kg probiotics (Diet D). The birds were disinfected by fumigation. Broiler chicks were fed with powder feed on the net. Chicks were reared from 1 day old and were allowed ad libitum access to both feed and water throughout the 42-day experimental period. The light regimen on day 1 was 23 L/1 D, which was gradually decreased to 14 L/10 D on day 6 and then maintained until the end of the experiment. The room temperature was 32 °C on day 1, which was gradually reduced to 21 °C on day 21 and then held. All chicks were vaccinated with the vaccines for Newcastle disease and infectious bursal disease, which were provided by Sanyi Animal Medicine Co., Ltd. (Dalian, China). Broiler feeding and management were implemented according to common requirements. Temperature, relative humidity, body weight, and feed consumption were daily recorded. The study was subjected to ethical review and approved by the Animal Experiment Committee of Anhui Science and Technology University.

2.4. Sample collection and treatment

On day 21 and day 42, 18 randomly selected broilers from each group were fasted for 12 h, weighed, and then slaughtered at a local commercial slaughterhouse. Six duplicates were arranged in the experiments. After dissection, breast muscle and thigh muscle were weighed with an electronic analytical balance (MS304S, Mettler Toledo, USA). Muscle paraffin sections were immediately prepared according to the following procedure: breast muscle with the size of 1.0 cm × 0.5 cm × 0.5 cm was cut and then subjected to tissue fixation with 4% paraformaldehyde in PBS, dehydration with graded concentrations of ethanol, xylene treatment, paraffin embedment, slicing (6- μ m-thick cross-sections), H&E staining, microscopic observation, and photography (BX51 and DP73, Olympus, Japan). The remaining breast muscle was used to determine muscle fiber diameter and meat quality.

2.5. Muscle fiber diameter measurement

Breast muscle (2.0 mm × 1.0 mm × 1.0 mm) was cut along the direction of the muscle fibers, cured at 4 °C for 24 h, and soaked in 20% nitric acid for 24 h. After breast muscle was placed on slide glass and 70% glycerol was added to the breast muscle, the muscle fibers were uniformly dispersed with the dissecting needle and covered with a cover glass. The diameter of the muscle fibers was then measured with a micrometer under microscope (BX51). In each sample, 30 muscle fibers were randomly measured.

2.6. Meat quality determination

2.6.1. Meat color determination

Within 45 min after dissection, a color analyzer (CR-410, Konica Minolta, Japan) was used to measure breast muscle

color values of L^* , a^* , and b^* . In each sample, 5 zones were selected and measured twice.

2.6.2. Determination of water-holding capability

Within 1 h after dissection, about 10 g of breast muscle was dried at 105 °C until the weight was constant. After cooling, dried muscle was weighed and water content (W_1) was calculated. At the same time, about 2 g of breast muscle (W_2) was weighed. Breast muscle was placed between 2 layers of medical gauze, cushioned with 18 layers of filter paper, and then subjected to the pressure of 68.66 KPa at 25 °C for 5 min. After the pressure was removed, breast muscle weight was measured. Water-holding capability (WHC) can be calculated according to Eq. (1):

$$\text{WHC} = [W_1 - (W_2 - W_3)] / W_1 \times 100\%. \quad (1)$$

2.6.3. Cooking loss

Within 1 h after dissection, breast muscle (W_4) was weighed, placed on an aluminum grill tray, placed in an oven (DHG-9030A, CHINCAN, China) preheated to 165 °C until the temperature at the center of the breast muscle reached 75 °C, and then taken out for cooling and weighing (W_5). Cooking loss can be calculated according to Eq. (2):

$$\text{Cooking loss} = W_5 / W_4 \times 100\%. \quad (2)$$

2.6.4. Shear force

The appropriate quantity of breast muscle was acquired and put in a plastic bag. A thermometer was placed in the center of the breast muscle sample. The meat sample and the thermometer in the plastic bag were put into a water bath at 80 °C. When the temperature at the center of the meat samples reached 72 °C, the samples were taken out and then cooled to room temperature. After meat surface-water was removed with filter paper, muscle pieces (2.0 cm × 1.0 cm) were cut along the vertical direction of the muscle fibers. In each sample, 2 muscle pieces were acquired and put on a tenderness meter (C-LM3B, Tenovo, China) to determine shear force.

2.6.5. Drip loss

Within 1 h after dissection, 2.0 g of breast muscle (W_6) was weighed. One end of the muscle sample was tied with cotton threads and loaded in a zip-lock bag along the vertical direction of the muscle fibers (the muscle sample did not touch the bag wall). After sealing the bags, the bags were suspended for 24 h at 4 °C and then weighed (W_7). Drip loss can be calculated according to Eq. (3):

$$\text{Drip loss} = (W_6 - W_7) / W_6 \times 100\%. \quad (3)$$

2.7. Data processing

The experiment was conducted as a completely randomized design with 6 replicates. All experimental data were

analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). The homogeneity of data variances was assessed by ANOVA using the general linear model procedure and means were separated by the least significant difference procedure. A significance value of $P < 0.05$ was considered as significant differences between treatments. The experimental unit was a pen. Experimental data are indicated as mean ± standard error.

3. Results

3.1. Breast muscle weight, thigh muscle weight, and organ index

The effects of probiotics on breast muscle weight, thigh muscle weight, and organ index of broilers are shown in Figure 1. On day 21, muscle weight showed no significant difference among the 4 groups ($P > 0.05$). Compared with Group A, Group C and Group D showed no significant difference in muscle index ($P > 0.05$), but breast and thigh muscle index in Group C was significantly lower by 14.52% and 5.5% than that in Group B ($P < 0.05$). On day 42, muscle weight and organ index in Groups B, C, and D were significantly higher than in Group A ($P < 0.01$ or $P < 0.05$). Breast muscle weight and thigh muscle weight in Group D were significantly increased by 31.9% and 35.94% ($P < 0.01$) and breast muscle index and thigh muscle index were significantly increased by 13.98% and 14.02% ($P < 0.05$). Compared with Group B, Groups C and D showed no significant difference in breast muscle weight, thigh muscle weight, or organ index ($P > 0.05$).

3.2. Muscle fiber diameter

The effects of probiotics on breast muscle fiber diameter of broilers are shown in Figure 2. On day 21, breast muscle fiber diameter showed no significant difference among the 4 groups ($P > 0.05$), but breast muscle fiber diameter in Group D was the largest. On day 42, muscle fiber diameters in Groups C and D were respectively 14.69% and 24.33% higher than in Group A ($P < 0.01$), but no significant difference was found between Group A and Group B ($P > 0.05$). Breast muscle fiber diameters in Groups C and D were respectively 7.75% and 16.81% higher than in Group B ($P < 0.05$). Breast muscle fiber diameter in Group D was 8.41% higher than in Group C ($P < 0.05$).

3.3. Muscle tissue structure

Structural changes in cross-section tissues of breast muscle in broilers are shown in Figures 3A–3H. On day 21, muscle fibers in Group A showed clear structure, neat arrangement, and obvious connective tissues among the muscle fibers (Figure 3A). There was much more connective tissue among the muscle fibers in Group B than in Group A (Figure 3C). Compared with breast muscle in Groups A and B, breast muscle in Groups C and D showed clear structure, increased cross-section, and significantly decreased connection tissues among muscle fibers

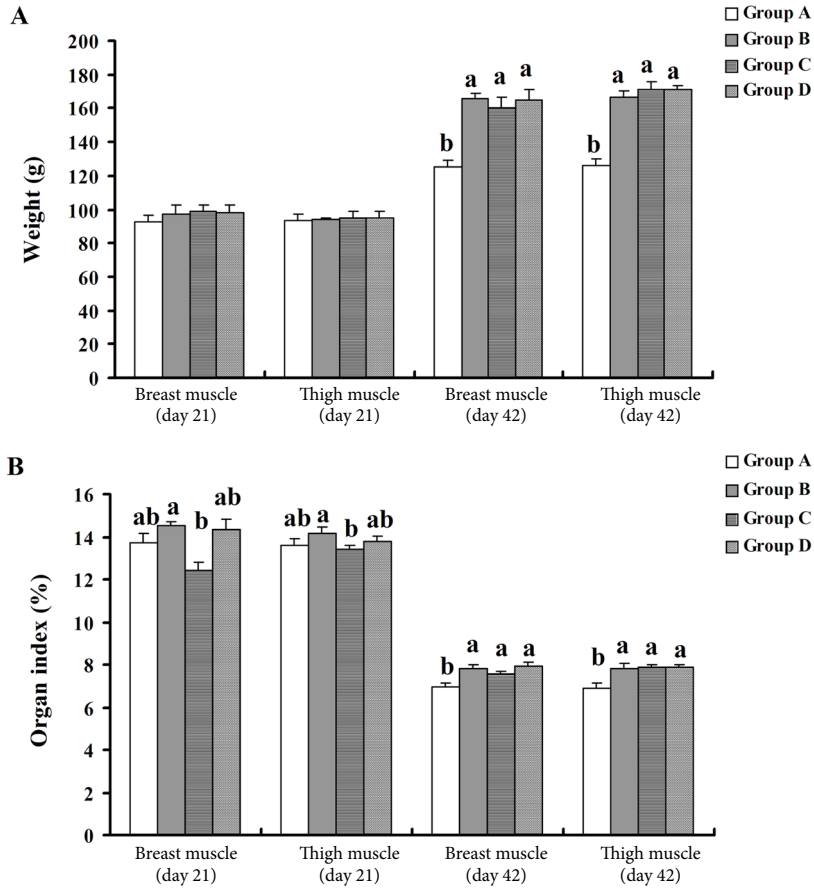


Figure 1. Effect of probiotics on weight (A) and organ index (B) of breast muscle and thigh muscle in broilers. Columns with different lowercase letters indicate $P < 0.05$.

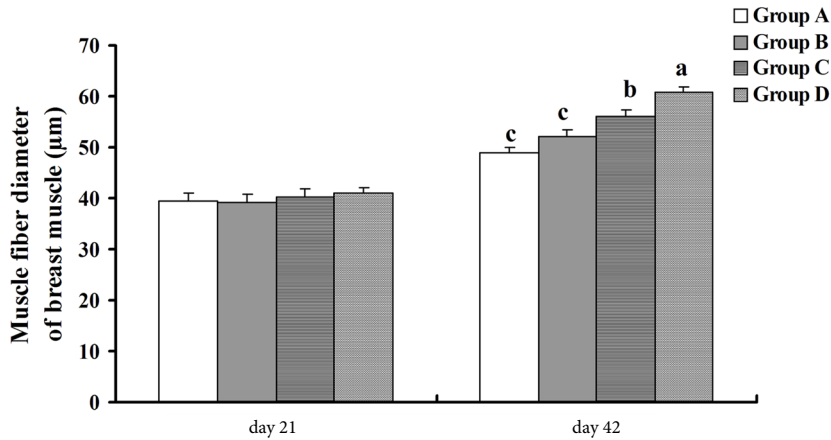


Figure 2. Effect of probiotics on muscle fiber diameter of breast muscle in broilers. Columns with different lowercase letters indicate $P < 0.05$.

(Figures 3E and 3G). On day 42, Group A showed neatly arranged breast muscle fiber and more connective tissues (Figure 3B). Compared with Group A, Group B increased cross-area of muscle fiber and decreased connective

tissues (Figure 3D). Breast muscle fibers in Groups C and D showed neat muscle arrangement. The cross-section of breast muscle fibers in Groups C and D was significantly larger than in Group A. Compared with Group B, Groups

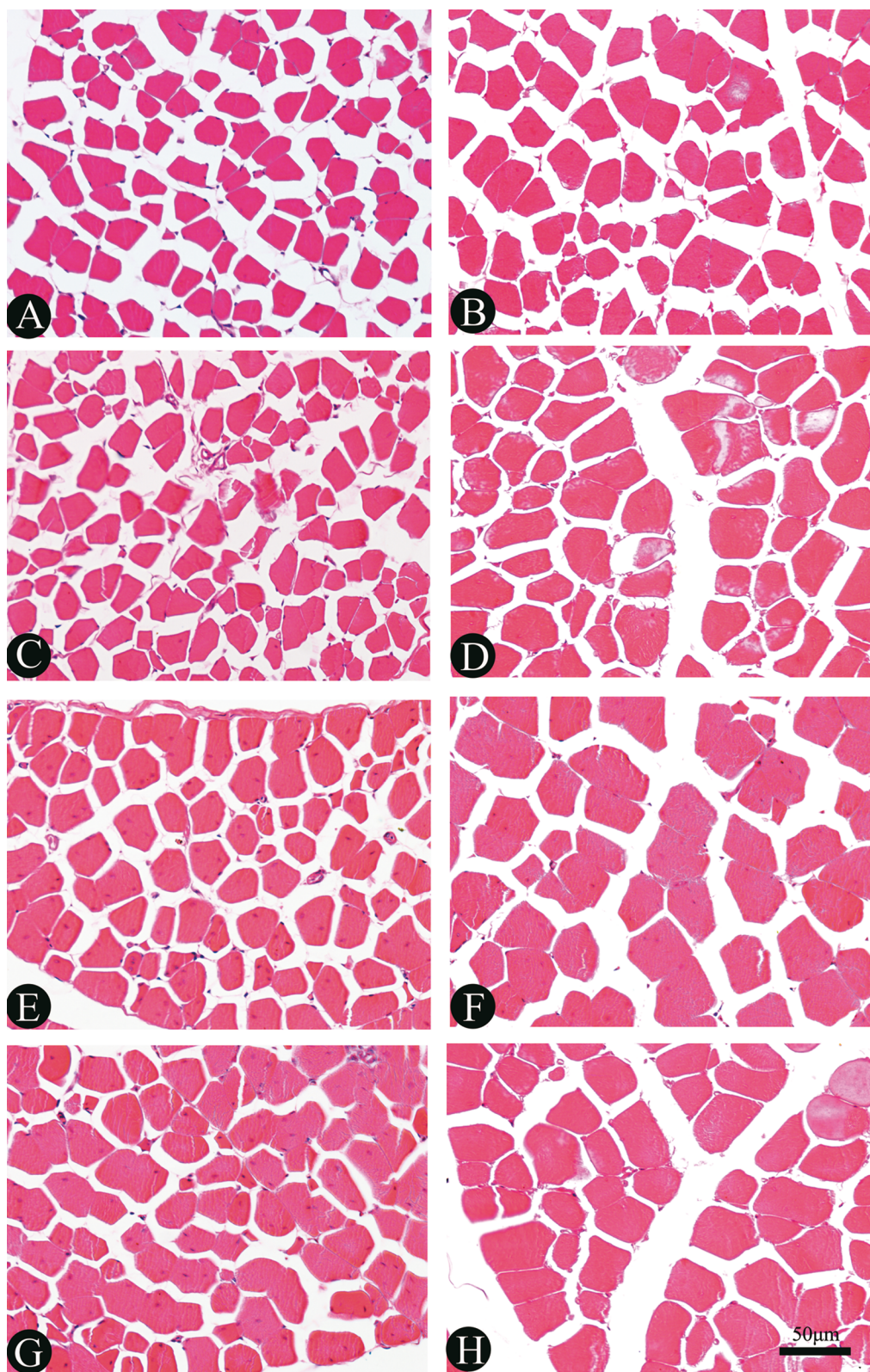


Figure 3. Effects of probiotics on the cross-section microstructure of breast muscle in broilers (H&E staining). A, C, E, and G: Cross-sections of breast muscle of 21-day-old broilers; B, D, F, and H: Cross-sections of breast muscle of 42-day-old broilers; A and B: Group A; C and D: Group B; E and F: Group C; G and H: Group D. Bar = 50 µm.

C and D showed no significant difference in the cross-section of breast muscle fibers. The connective tissues among muscle fibers in Groups C and D were significantly less than those in Group A, but more than those in Group B (Figures 3F and 3H).

3.4. Breast meat color

The effects of probiotics on breast meat color of broilers are provided in Table 2. On day 21, breast meat color values of L*, a*, and b* showed no significant difference among the 4 groups (P > 0.05). On day 42, breast meat color values of L* or a* also showed no significant difference among the 4 groups (P > 0.05), but breast meat color values of b* in Groups C and D were respectively 45.27% and 56.55% lower than those in Group A (P < 0.05) and 38.09% and 50.84% lower than those in Group B (P < 0.05).

3.5. Water-holding capacity, drip loss, cooking loss, and shear force of breast muscle

The effects of probiotics on water-holding capacity, drip loss, cooking loss, and shear force of breast muscle of broilers are provided in Table 3. On day 21, water-holding capacity, drip loss, cooking loss, and shear force of breast muscle showed no significant difference among the 4 groups (P > 0.05), and Group D showed the highest water-holding capacity and the lowest shear force of breast muscle. On day 42, water-holding capacity of breast muscle showed no significant difference among the 4 groups (P > 0.05). On day 42, drip loss levels of breast muscle in Groups B, C, and D were 33.16%, 28.74%, and 40.23% lower than in Group A, respectively (P < 0.05), and no significant difference was found among Groups B, C, or D (P > 0.05). On day 42, cooking loss and shear force of breast muscle in Groups D were 7.83% and 11.83% lower

Table 2. Effects of probiotics on meat color of breast muscle in broilers.

Age	Item	Group A	Group B	Group C	Group D
Day 21	L*	-34.76 ± 1.25	-36.45 ± 1.86	-36.14 ± 1.48	-36.58 ± 1.52
	a*	-0.77 ± 0.86	-0.18 ± 1.07	-0.62 ± 0.96	-0.52 ± 0.38
	b*	5.34 ± 1.20	7.53 ± 0.86	5.13 ± 0.94	5.31 ± 0.40
Day 42	L*	-34.58 ± 0.71	-36.64 ± 1.52	-36.30 ± 1.00	-36.75 ± 0.61
	a*	-12.76 ± 0.77	-11.63 ± 1.13	-10.64 ± 0.88	-7.77 ± 0.39
	b*	6.03 ± 0.36 ^a	5.33 ± 0.35 ^a	3.30 ± 0.33 ^b	2.62 ± 0.19 ^b

^{ab}: Different superscripts within rows indicate significance (P < 0.05).

Table 3. Effects of probiotics on water-holding capability, drip loss, cooking loss, and shear force of breast muscle in broilers.

Age	Item	Group A	Group B	Group C	Group D
Day 21	Water-holding capability (%)	43.50 ± 4.15	42.27 ± 3.77	44.32 ± 3.11	44.43 ± 5.16
	Drip loss (%)	2.26 ± 0.19	2.62 ± 0.13	2.13 ± 0.12	2.16 ± 0.21
	Cooking loss (%)	32.20 ± 1.08	32.47 ± 0.53	30.29 ± 0.64	30.40 ± 0.92
	Shear force (kg F)	1.49 ± 0.13	1.49 ± 0.06	1.37 ± 0.07	1.35 ± 0.03
Day 42	Water holding capability (%)	43.30 ± 4.21	45.73 ± 4.38	49.15 ± 4.08	52.35 ± 4.10
	Drip loss (%)	2.61 ± 0.13 ^a	1.96 ± 0.08 ^b	1.86 ± 0.11 ^b	1.56 ± 0.07 ^b
	Cooking loss (%)	70.14 ± 0.75 ^a	71.20 ± 0.72 ^a	68.00 ± 0.85 ^{ab}	64.58 ± 0.93 ^b
	Shear force (kg F)	1.69 ± 0.04 ^a	1.67 ± 0.04 ^a	1.58 ± 0.03 ^{ab}	1.49 ± 0.05 ^b

^{ab}: Different superscripts within row indicate significance (P < 0.05).

than in Group A and were 9.3% and 10.78% lower than in Group B, respectively ($P < 0.05$). On day 42, compared with Groups A, B, and D, Group C showed no significant difference in cooking loss or shear force of breast muscle ($P > 0.05$) and there was no significant difference between Group A and Group B ($P > 0.05$).

4. Discussion

4.1. Effects of probiotics on growth and development of broiler muscles

Animal muscle tissue development involves the generation of new muscle fibers and the elongation and augmentation of existing muscle fibers. The number of muscle fibers in animals is basically stable before birth. Muscle tissue development after birth mainly involves the elongation and augmentation of existing muscle fibers (12). Dietary nutrient levels and intestinal absorption of nutrients directly affect muscle tissue development (13). Favorable symbiosis of beneficial intestinal microflora in the intestinal tracts determines intestinal mucosal structure, intestinal function, and intestinal absorption of nutrients (14). In this experiment, the adopted 3 probiotics, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus natto*, have different effects on animal growth and development. As one of the cecal mucosa bacteria of broilers (15), *Bacillus subtilis* can consume free oxygen in the intestinal environment, promote the growth of beneficial intestinal anaerobic bacteria, and enhance intestinal digestive function (16). *Bacillus licheniformis* can produce antimicrobial substances, kill pathogens, promote the growth of beneficial bacteria, and regulate intestinal microflora balance (17). *Bacillus natto* can produce a variety of digestive enzymes, enhance digestion capability, and improve the feed conversion ratio (18). In this study, on day 21, Groups C and D showed no significant difference in growth performance indices except that the connective tissues among muscle fibers were decreased. On day 42, Groups C and D showed significantly increased growth performance indices (including breast muscle weight, thigh muscle weight, breast muscle index, thigh muscle index, breast muscle fiber diameter, and muscle fiber cross-area) and decreased connective tissues among muscle fibers. Group B also showed significantly increased weight and organ index of breast and thigh muscle, but no significant effect on breast muscle fiber diameter was found. The current results indicate that probiotics could enhance intestinal digestion and nutrient absorption and further promote muscle tissue development through improving the intestinal microflora and composition.

4.2. Effects of probiotics on broiler meat quality

The immoderate application of antibiotics and synthetic

chemical additives in the poultry feeding industry has led to increasingly prominent drug residues in poultry products and has seriously affected the quality, safety, and consumption of poultry products (19). It is urgent to develop new green feed additives for poultry feeding to decrease the consumption of antibiotics and synthetic drugs. Animal tests showed that probiotics could improve animal production performance, enhance immune function, adjust the intestinal microflora, and significantly improve the quality of animal muscles (20). Meat quality assessment indicators include meat color, pH, water-holding capacity, drip loss, cooking loss, and shear force (21). Meat color is an important apparent meat quality indicator (22). Meat color change affects pH and water-holding capability of muscle and alters drip loss, cooking loss, and shear force of muscle (23). Dietary supplementation with single-strain or multistrain probiotics (*Bacillus subtilis* and *Bacillus licheniformis*) can significantly decrease breast muscle color value of b^* and cooking loss and increase color value of a^* , while such supplementation showed no significant effect on water-holding capability or shear force (24). The above results indicate that *Bacillus subtilis* and *Bacillus licheniformis* could change meat color and affect muscle tenderness. In Groups C and D, respectively fed with 2 basal feeds supplemented with 200 mg/kg probiotics and 400 mg/kg probiotics, meat color, water-holding capability, drip loss, cooking loss, and shear force of 21-day-old broilers also showed no significant difference, while breast muscle color value of b^* , drip loss, cooking loss, and shear force were significantly decreased in 42-day-old broilers. The supplementation of 400 mg/kg probiotics showed significant improvement in meat quality. The above results indicate that probiotics could affect muscle color and improve muscle tenderness because *Bacillus natto* in probiotics could produce a variety of digestive enzymes.

In conclusion, basal diets supplemented with 200 mg/kg or 400 mg/kg probiotics could promote the development of breast and thigh muscle tissues of 42-day-old broilers and improve breast muscle color, tenderness, and meat quality. The supplementation of 400 mg/kg probiotics showed significant meat quality improvements.

Acknowledgments

This study was financially supported by the Natural Science Fund of Anhui, China (1308085MC50, 1208085QC68); the National Spark Program Projects (2012UA710027); the Talent Fund of Anhui Science and Technology University, China (ZRC2013354); the Youth Science Foundation of Anhui Science and Technology University (ZRC2013346); and the Key Discipline Construction Program of Anhui Science and Technology University, China (AKXK20101-2).

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