



Effect of *Bacillus subtilis* Natto on Meat Quality and Skatole Content in TOPIGS Pigs

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ABSTRACT: This study investigated the effect of *Bacillus subtilis* (*B. subtilis*) natto on meat quality and skatole in TOPIGS pigs. Sixty TOPIGS pigs were randomly assigned to 3 groups (including 5 pens per group, with 4 pigs in each pen) and fed with basic diet (control group), basic diet plus 0.1% *B. subtilis* natto (B group), and basic diet plus 0.1% *B. subtilis* natto plus 0.1% *B. coagulans* (BB group), respectively. All pigs were sacrificed at 100 kg. Growth performance, meat quality, serum parameters and oxidation status in the three groups were assessed and compared. Most parameters regarding growth performance and meat quality were not significantly different among the three groups. However, compared with the control group, meat pH₂₄, fat and feces skatole and the content of *Escherichia coli* (*E. Coli*), *Clostridium*, NH₃-N were significantly reduced in the B and BB groups, while serum total cholesterol, high density lipoprotein, the levels of liver P450, CYP2A6, and CYP2E1, total antioxidant capability (T-AOC) and glutathione peroxidase and *Lactobacilli* in feces were significantly increased in the B and BB groups. Further, the combined supplementation of *B. subtilis* natto and *B. coagulans* showed more significant effects on the parameters above compared with *B. subtilis*, and *Clostridium*, and NH₃-N. Our results indicate that the supplementation of pig feed with *B. subtilis* natto significantly improves meat quality and flavor, while its combination with *B. coagulans* enhanced these effects. (**Key Words:** *Bacillus subtilis* Natto, *Bacillus coagulans*, Meat Quality, Skatole, TOPIGS Pig)

INTRODUCTION

Customers tend to prefer high quality of meat. Meat quality is mostly determined by appearance, tenderness, flavor, fat content, water-holding capacity, oxidative stability and uniformity (Rosenvold and Andersen, 2003; Lomiwes et al., 2014). Due to different food culture and cooking habits, customers worldwide mostly focus on meat flavor that is affected by meat composition and odors (Zahn et al., 1997). These odors are produced as a result of anaerobic degradation of materials present in feces (Zahn et al., 1997). Skatole (3-methylindole) is one of the most

malodorous compounds identified in swine feces and is also an off-flavor component of pig meat (Strathe et al., 2013).

Bacillus subtilis (*B. subtilis*) natto is usually used to produce salt-free fermented soy food. It has been demonstrated that *B. subtilis* natto is activated to strengthen beneficial intestinal flora, inhibit harmful intestinal bacteria, produce protein and starch degradation enzymes, and increase the production of B-complex vitamins (Nagai, 2012; Bhat et al., 2013). *B. subtilis* natto has been widely used as a probiotic in animal feeds (Giang et al., 2011). It has been demonstrated that the addition of *B. subtilis* improved growth performance, nutrient digestibility and meat quality, and upregulated lipid metabolism in subcutaneous fat of growing-finishing pigs (Meng et al., 2010; Cui et al., 2013), but the relationship between *B. subtilis* and skatole is unclear.

Further, Sheng et al. (2015) reported that *B. subtilis* natto reduced the content of skatole in pig feces and increased the content of *Streptococcus alactolyticus* and

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Lactobacillus amylovorus (*L. amylovorus*) in fermented broths. *S. alactolyticus* and *L. amylovorus* are lactic acid bacteria in the intestine (Rinkinen et al. 2004; Eom et al., 2009). Supplementing lactic acid bacteria in the feed has the potential to reduce skatole and improve meat quality (Wajda et al., 2010). *B. coagulans* is a probiotic known as “spore-forming lactic acid bacteria” and shows resistance to gastric acid and high temperature during feed granulation. *B. coagulans* has been found to stimulate productive performance and improve meat quality in chickens (Zhou et al., 2010). Previous studies mostly examined the efficacy of single probiotic in animal feed on growth performance and meat quality (Zhou et al., 2010; Giang et al., 2011; Nagai, 2012; Bhat et al., 2013). The combined efficacy of two or multiple probiotics, such as *B. subtilis* natto and *B. coagulans*, in improving animal growth performance and meat quality has been rarely studied.

The intramuscular fat has been considered as one of the important factors for meat marbling, tenderness, flavor and water-holding capacity (Faucitano et al., 2004). TOPIGS pigs are characterized by fast growth, low fat and high lean growth, and therefore, are good models for studying meat quality. In the present study, we investigated the role of *B. subtilis* natto and combined probiotics (*B. subtilis* natto and *B. coagulans*) in improving meat quality and reducing skatole in TOPIGS pigs.

MATERIALS AND METHODS

Animals and materials

Sixty neutered healthy TOPICS growing pigs with similar sex ratio, dates of birth, parity and weight (30.0 ± 0.5 kg) were purchased from Jinan Lv'an Food Corp. (Jinan, China). *B. subtilis* natto and *B. coagulans* at a concentration of 1×10^9 cfu/g were provided by the Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences (Jinan, China). The recipe of basic diet was referred to as the Feeding Standard of Swine (NY/T65-2004) and produced by Jinan Lv'an Food Corp. It was mostly composed of corn and soybean meal (Table 1). All animal procedures were approved by the ethics committee of Shandong Academy of Agricultural Sciences.

All pigs were randomly assigned to three groups, with each group comprising 5 individual pen replicates including 4 pigs per pen, and fed with basic diet (control group), basic diet plus 0.1% *B. subtilis* natto (B group), and basic diet plus 0.1% *B. subtilis* natto plus 0.1% *B. coagulans* (BB group), respectively. All pigs were housed in an environmentally controlled room at a temperature of 25°C and 60% humidity and managed by a designated person. All pigs were allowed access to feed and water *ad libitum* throughout the experimental period. The body weight (BW) before and after the experiment were measured to calculate

Table 1. Nutritional composition of the basal diet

Items	Growth phase	
	30 to 60 kg	60 to 100 kg
Ingredients		
Corn (%)	68.2	70.8
Soybean meal (%)	21	16.0
Wheat bran (%)	8	10.5
Salt (%)	0.3	0.3
L-lysine hydrochloride (%)	0.1	0.1
Calcium carbonate (%)	0.9	0.9
Calcium hydrophosphate (%)	0.5	0.4
Vitamin-mineral premix ¹ (%)	1.0	1.0
Total	100	100
Nutrient levels		
Metabolizable energy (MJ/kg)	12.88	12.88
Crude protein (%)	16.0	14.6
Calcium (%)	0.58	0.49
Total Phosphorous (%)	0.53	0.46
Lysine (%)	0.79	0.71
Methionine (%)	0.24	0.22
Threonine (%)	0.55	0.49

¹Mixture contained the following components: 100 mg/kg iron (as iron sulfate), 30 mg/kg copper (as copper sulfate), 100 mg/kg zinc (as zinc sulfate), 20mg/kg manganese (as manganese sulfate), 0.03 mg/kg selenium (as sodium selenite), 0.04 mg/kg iodine (as potassium iodate), 1,650 IU/kg vitamin A (trans-retinyl acetate), 280 IU/kg vitamin D₃, 22 IU/kg vitamin E (DL-alpha-tocopherol acetate), 2 mg/kg vitamin K₃, 20 mg/kg niacin, 15 mg/kg pantothenic acid, 1.0 mg/kg folic acid, 0.03 mg/kg vitamin B₁₂, 2.4 mg/kg vitamin B₁, 5 mg/kg vitamin B₂, 2.4 mg/kg vitamin B₆. The carrier was zeolite.

the average daily gain (ADG). The weather, feed intake per pen (n = 5) and healthy conditions were recorded every day. The average daily feed intake (ADFI) and feed intake/gain (F/G) were calculated.

Sample collection

After fasting overnight, 10 mL of venous blood was collected from the inferior vena cava of all pigs and centrifuged at 1,000 g for 5 min to collect serum (n = 20). All pigs were sacrificed at a BW of ~100 kg at Jinan Lv'an Food Corp. and fragmented according to the conventional procedure (n = 20). The backfat thickness and loin eye area were measured. A 20 to 30 cm of longissimus from the last third or fourth rib to back was separately assessed to measure meat color, water-holding capacity, pH values at 1 and 24 hrs (pH₁ and pH₂₄), and intramuscular fat content. Twenty grams of back fat and 5 g of liver tissue were collected and frozen at -18°C. The feces from the rectum of two pigs in each pen were collected to analyze skatole and bacterial composition (n = 10 in each group). All sample collection was completed within 30 min.

Measurement of meat, serum, liver and feces parameters

Backfat thickness and loin eye area were measured

Table 2. Effect of *Bacillus subtilis* natto on growth performance

	IBW (kg/h)	FBW (kg/h)	ADFI (kg/d·h)	ADG (kg/d·h)	F/G (kg/kg)
Number	20	20	5	20	5
Control	30.09±0.44	101.55±8.70	2.22±0.11	0.85±0.09	2.61±0.15
B group ¹	30.13±0.41	103.39±10.61	2.26±0.10	0.87±0.07	2.59±0.13
BB group ¹	30.04±0.39	103.62±11.24	2.27±0.14	0.88±0.10	2.59±0.10
F-value	0.10	0.54	0.35	0.26	0.08

Bacillus subtilis, *B. subtilis*; IBW, initial body weight; FBW, final body weight; ADFI, average daily feed intake; ADG, average daily gain; F/G, feed intake/gain.

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

according to the protocol described by Chen and Wang (1997). The meat parameters including color, water-holding capacity, pH₁, pH₂₄ and intramuscular fat content were measured according to "Determination of pig meat quality technical specifications" (NY/T821-2004). The fatty acids were measured by gas chromatography at Food Detection Center of the Ministry of Agriculture following "Meat and meat products-Determination of fatty acids" specifications (GB9695.2-2008). The levels of serum triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), total antioxidant capability (T-AOC) and glutathione peroxidase (GSH-PX) were measured by the kits supplied by Nanjing Jiancheng Biotechnology Corp. (Nanjing, China). The serum and liver cytochrome P450 (P450), cytochrome oxidase 2A6 (CYP2A6) and cytochrome oxidase 2E1 (CYP2E1) were measured using the enzyme-linked immunosorbent assay kits from Wuhan Beinglay Biotechnology Corp. (Wuhan, China). The liver tissue parameters were measured using the supernatant obtained by homogenizing and centrifuging 1.0 g liver tissue in 9.0 mL cold physiological saline. The skatole levels were measured in the supernatant following homogenization and centrifugation of 2.0 g fat or feces in 18.0 mL cold methanol. The skatole level in the supernatant was measured by high-performance liquid chromatography

at Food Detection Center of Ministry of Agriculture (Jinan, China). The numbers of *Lactobacilli*, *E. coli*, and *Clostridium* were assessed by plate count, while the NH₃-N level in feces was measured by Nessler colorimetry.

Statistical analysis

The SAS 9.2 program (SAS Institute, Cary, NC, USA) was used for statistical analyses. All data were presented as mean±standard error of the mean and analyzed by one-way analysis of variance. The Bonferonni correction was used for multiple comparisons. Statistical significance was defined as p<0.05.

RESULTS

Effect of *B. subtilis* natto on growth performance and meat quality

There were no significant differences in initial BW and final BW, ADG, ADFI, and F/G among the three groups (p>0.05, Table 2).

The meat parameters including backfat thickness, loin eye area, color, water-holding capacity, intramuscular fat content, palmitic acid, stearic acid, oleic acid, and linoleic acid were not significantly different between the three groups (p>0.05, Tables 3 and 4). However, compared with

Table 3. Effect of *Bacillus subtilis* natto on meat parameters

	Backfat thickness (mm)	Loin eye area (cm ²)	Color L value	Water-holding capacity (%)	pH ₁	pH ₂₄	Fat skatole (µg/kg)
Control	11.30±0.24	44.87±1.55	48.18±2.33	2.79±1.87	6.20±0.14	5.47±0.06 ^a	14.86±1.27 ^a
B group ¹	10.00±0.21	43.77±1.74	48.71±2.57	2.84±2.21	6.23±0.13	5.51±0.04 ^b	13.61±2.09 ^b
BB group ¹	10.77±0.40	43.60±2.37	46.99±3.26	2.88±1.96	6.29±0.09	5.55±0.08 ^c	12.42±1.95 ^c
F-value	2.78	1.34	1.88	1.29	2.50	8.77	14.28

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

^{a,b,c} Means within same column with different superscript letters are significantly different (p<0.05). n = 20.

Table 4. Effect of *Bacillus subtilis* natto on fatty acids (%) in the longissimus

	Intramuscular fat	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid
Control	1.56±0.57	23.62±1.06	12.27±0.96	42.87±4.34	8.83±1.67
B group ¹	1.85±0.49	24.23±0.94	12.33±0.68	45.70±1.48	6.28±1.41
BB group ¹	1.62±0.44	24.58±0.70	12.25±0.65	45.23±2.10	6.23±1.39
F-value	0.59	1.48	0.12	1.63	2.41

n = 20.

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

the control group, pH₂₄ in meat was significantly increased while the level of fat skatole was significantly reduced in the B and BB groups ($p < 0.05$, Table 3). There were no significant differences in pH₂₄ and fat skatole between B and BB groups ($p > 0.05$, Table 3).

Effect of *B. subtilis* natto on serum parameters

Compared with the control group, the levels of TG and LDL were not changed ($p > 0.05$) while the TC and HDL levels were significantly reduced in the B and BB groups ($p < 0.05$, Table 5). Further, the levels of TC and HDL in the BB group were significantly lower than in the B group ($p < 0.05$, Table 5).

Effect of *B. subtilis* natto on the oxidative parameters of serum and liver

To examine the effects of *B. subtilis* natto and *B. coagulans* on oxidative status in pigs, we first assessed the levels of serum T-AOC, GSH-PX, P450, CYP2A6, and CYP2E1 and observed that the levels of serum T-AOC and GSH-PX were significantly increased in the B and BB groups compared with the control group ($p < 0.05$ or $p < 0.01$, Table 5). Further, serum T-AOC in the BB group was significantly higher than in the B group ($p < 0.05$, Table 6). The serum P450, CYP2A6, and CYP2E1 levels were very low and not significantly different among the three groups ($p > 0.05$, Table 6).

We also examined the levels of P450, CYP2A6, and CYP2E1 in the liver and found that the levels of P450, CYP2A6, and CYP2E1 in the B and BB groups were significantly higher than those in the control group ($p < 0.05$, Table 6). However, there were no significant differences in

Table 5. Effect of *Bacillus subtilis* natto on serum parameters (mmol/L)

	TC	TG	HDL	LDL
Control	2.33±0.57 ^a	0.27±0.13	1.64±0.53 ^a	0.48±0.39
B group ¹	2.21±0.87 ^b	0.23±0.09	1.34±0.33 ^b	0.45±0.47
BB group ¹	2.07±0.55 ^c	0.22±0.09	1.06±0.37 ^c	0.42±0.36
F-value	6.56	1.59	6.01	0.81

TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein.

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

^{a,b,c} Means within same column with different superscript letters are significantly different ($p < 0.05$). n = 20.

the levels of hepatic P450, CYP2A6, and CYP2E1 between B and BB groups ($p > 0.05$, Table 6).

Effect of *B. subtilis* natto on skatole production and bacterial composition in the feces

Compared with the control group, the *Lactobacilli* in the feces were increased while the numbers of *E. coli* and *Clostridium* and the levels of NH₃-N and skatole in feces were significantly decreased in the B and BB groups ($p < 0.05$, Table 7). There were significant differences in the numbers of *Lactobacillus*, and *Clostridium*, and the levels of NH₃-N in feces between B and BB group except *E. coli* and skatole ($p < 0.05$, Table 7).

DISCUSSION

Meat quality is affected by many different factors, such as intramuscular fat, skatole level in meat, etc. However, the production of skatole is associated with the intestinal

Table 6. Effects of *Bacillus subtilis* natto on serum and liver oxidative parameters

	Serum					Liver		
	T-AOC (U/L)	GSH-PX (U/mL)	P450 (ng/mL)	CYP2A6 (ng/mL)	CYP2E1 (ng/mL)	P450 (ng/mL)	CYP2A6 (ng/g)	CYP2E1 (ng/g)
Control	4.37±0.21 ^c	366.59±15.48 ^b	0.22±0.04	0.12±0.01	0.16±0.02	1.85±0.13 ^b	1.02±0.09 ^b	1.32±0.14 ^b
B group ¹	5.88±0.55 ^b	382.31±9.77 ^a	0.26±0.05	0.14±0.02	0.17±0.04	2.07±0.17 ^a	1.15±0.11 ^a	1.49±0.19 ^a
BB group ¹	8.10±0.45 ^a	382.44±12.02 ^a	0.26±0.04	0.14±0.03	0.17±0.03	2.09±0.19 ^a	1.18±0.18 ^a	1.51±0.21 ^a
F-value	9.77	2.89	1.08	1.24	0.88	4.31	5.07	5.78

T-AOC, total antioxidant capability; GSH-PX, glutathione peroxidase; P450, cytochrome P450; CYP2A6, cytochrome oxidase 2A6; CYP2E1, cytochrome oxidase 2E1.

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

^{a,b,c} Means within same column with different superscript letters are significantly different ($p < 0.05$). n = 20.

Table 7. Effect of *Bacillus subtilis* natto on skatole production and fecal bacterial composition

	<i>Lactobacillus</i> (log ₁₀ CFU/g)	<i>Escherichia coli</i> (log ₁₀ CFU/g)	<i>Clostridium</i> (log ₁₀ CFU/g)	NH ₃ -N (g/kg)	Skatole (mg/kg)
Control	5.74±0.12 ^c	5.38±0.11 ^a	5.55±0.09 ^a	1.64±0.16 ^a	23.66±3.33 ^a
B group ¹	7.06±0.09 ^b	5.14±0.14 ^b	4.92±0.08 ^b	0.71±0.18 ^b	18.39±2.19 ^b
BB group ¹	7.44±0.11 ^a	5.02±0.13 ^b	4.76±0.08 ^c	0.55±0.12 ^c	17.21±2.09 ^b
F-value	52.84	18.98	49.31	29.77	6.45

¹ B group, plus *B. subtilis* natto group; BB group, plus *B. subtilis* natto and *B. coagulans* group.

^{a,b,c} Means within same column with different superscript letters are significantly different ($p < 0.05$). n = 10.

bacterial composition and the enzymes secreted by the intestine. Thus, intestinal bacterial composition is one of the key factors that affect meat quality. In the present study, we observed that supplementation of *B. subtilis* natto in pig feed significantly changed the bacterial composition, increased CYP2E1 level in the liver and decreased skatole content in the fat and feces. These data indicate that *B. subtilis* natto supplementation of pig feed significantly improved meat quality.

Skatole is a malodorous chemical in pig meat and manure and is as an off-flavor component (referred to as "boar taint") (Strathe et al., 2013). Further, skatole has been found to pollute the environment and cause acute bovine pulmonary edema and emphysema (Linden et al., 1996). In this study, we demonstrated that pig feed supplementation using *B. subtilis* natto significantly decreased the skatole content of the fat and feces. This result is supported by two major findings. First, compared with the control group, reduced number of *Clostridium* in the feces was consistent with lower skatole content in the fat and feces in the B and BB groups. It has been demonstrated that *Clostridium* in the feces is involved in the transformation of tryptophan to skatole (Whitehead et al., 2008; Doerner et al., 2009). Second, we observed increased levels of CYP2A and CYP2E1 in the liver from B and BB groups, indicating increased metabolism of skatole in the B and BB groups. CYP2A and CYP2E1 in the liver are two important proteinases for the degradation of skatole (Zamaratskaia et al., 2006; Wiercinska et al., 2012). However, the mechanisms underlying reduction in the number of *Clostridium* and increased CYP2A6 and CYP2E1 levels in the liver by *B. subtilis* natto and *B. coagulans* need further investigation. Similar to CYP2A6 and CYP2E1, T-AOC and GSH-PX are two major biomarkers of oxidative status in the body. Here we also observed that *B. subtilis* natto supplementation of pig feed significantly increased serum T-AOC and GSH-PX, but it is unclear whether *B. subtilis* natto increased the antioxidant function via natto kinase and catalase (Hosoi et al., 2000; Yongjun et al., 2011).

In the present study, we also observed that the supplementation of *B. subtilis* natto in pig feed significantly decreased serum TC and increased serum HDL although not supported by any evidence. The result may be related to P450 or natto kinase because P450 and its family members including CYP8B, CYP4A, and CYP7A1 are involved in the cholesterol metabolism (Norlin and Wikvall, 2007; Lundell and Wikvall, 2008). Further, the combination of natto kinase and red yeast rice decreased the TC level in the patients with high blood lipids (Yang et al., 2009). Interestingly, the *B. subtilis* natto supplementation failed to increase growth performance of pigs, in which the FBW, ADG, F/G backfat thickness, loin eye area, meat color, water-holding capacity and intramuscular fat were very

similar among the three groups. These findings are not consistent with previous study in which dietary supplementation of *B. subtilis* increased growth performance throughout the experiment (Meng et al., 2010). The discrepancy between two studies may be caused by different pig species and *B. subtilis* resources. Additionally, the *B. subtilis* natto supplementation increased meat pH₂₄ value, which is related to anaerobic glycolysis in meat.

More importantly, the combined supplementation of *B. subtilis* natto and *B. coagulans* resulted in similar or better results in improving meat quality and antioxidant capacity and reducing skatole content compared with *B. subtilis* natto alone. These data suggest that *B. subtilis* natto and *B. coagulans* may exert synergistic effects in the pigs.

However, the study limitations are as follows. First, all parameters were measured only at the endpoint. The selection of endpoint at 100 kg may be a little arbitrary for evaluating growth performance and meat quality because growth performance is decreased and fat deposition is increased at the late stage of pig growth. Therefore, the delayed endpoint may affect the evaluation of growth performance. Second, the effects of *B. coagulans* in animal feed have been rarely studied, so it will be interesting to set an individual *B. coagulans* group.

In conclusion, *B. subtilis* natto supplementation of pig feed significantly improves meat quality, increases antioxidant function and reduces skatole production. Further, the combined supplementation of *B. subtilis* natto and *B. coagulans* has better effects.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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