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Effects of different *Bacillus licheniformis* and *Bacillus subtilis* ratios on nutrient digestibility, fecal microflora, and gas emissions of growing pigs

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Abstract

The objective of this study was to evaluate the effects of different mixing ratios of *Bacillus licheniformis* and *Bacillus subtilis* in diets on nutrient digestibility, fecal microflora, and odor gas emissions of growing pigs. A total of four crossbred ([Landrace × Yorkshire] × Duroc) barrows with average body weight (BW) of 41.2 ± 0.7 kg were randomly allotted four diets over four periods in a 4 × 4 Latin square design. Treatments were as follows: Control (CON, basal diet), CON + 0.2% probiotic complex (L4S6, *B. licheniformis* and *B. subtilis* at a 4:6 ratio), CON + 0.2% probiotic complex (L5S5, *B. licheniformis* and *B. subtilis* at a 5:5 ratio), CON + 0.2% probiotic complex (L6S4, *B. licheniformis* and *B. subtilis* at a 6:4 ratio). Dietary probiotic supplementation showed higher crude protein (CP) digestibility values and lower *Escherichia coli* counts in fecal samples than the CON group ($p < 0.05$). There was no significant difference in NH_3 or H_2S emission until day 3. The positive effect of H_2S and NH_3 emissions was detected earlier with the L4S6 and L5S5 compared to the L6S4, which had a lower ratio of *B. subtilis*. Both the L4S6 and L5S5 probiotic complexes significantly decreased the fecal H_2S and NH_3 emission in days 4 and 6 ($p < 0.05$). On day 7, all probiotic complexes decreased ($p < 0.05$) H_2S and NH_3 emissions than the CON group. Our results agreed that the dietary supplementation of *Bacillus licheniformis* and *Bacillus subtilis* complexes in growing pigs can significantly improve CP digestibility and reduce fecal *E. coli* counts, NH_3 and H_2S emissions. Notably, the higher mixing ratio of *Bacillus subtilis* in probiotic supplementation is more effective in reducing the odor of manure.

Keywords: *Bacillus subtilis*, *Bacillus licheniformis*, Probiotic, Nutrient digestibility, Odor gas emission, Growing pig

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No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

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Ethics approval and consent to participate
The experimental protocol was approved (CBNUA-1619-21-02) by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea.

INTRODUCTION

Antibiotics and chemical therapeutics as feed additives have been used constantly in the pig industry for economic benefits, improving feed efficiency [1]. Nevertheless, due to the increases of antibiotic-resistant pathogens, many countries such as Europe, China, Korea, and the United States have banned the use of antibiotics [2–5]. In addition, as a result of high nutrient values in feed for high growth performance, the pig industry has faced minimizing acute environmental problems such as harmful gas emissions [6–8]. Furthermore, fermentation of undigested dietary proteins and endogenous proteins in the large intestine produces toxic metabolites. The metabolites contribute to compromise epithelial integrity and promote intestinal disorders such as postweaning diarrhea [9–11].

Probiotic supplementation has been used to overcome the above-mentioned problems [12–14]. Probiotics can accelerate the breakdown of the carbohydrates which are resistant to digestion, thus it encourages the use of dietary fiber as a prebiotic substance, and promotes the substrate availability of colonic microbiota, and its population [15]. It has been extensively studied probiotics are able to support growth performance, gut micro-environment, feed utilization efficiency, immune system, and gastrointestinal tract (GIT) diseases for farm animals [16,17]. Currently, the main groups of probiotics, commonly used in animal feeds are lactic acid bacteria, yeast, and *Bacillus* [18]. Among them, *Bacillus*-based probiotics, especially *Bacillus subtilis* and *Bacillus licheniformis,* are widely used as their spore-forming properties facilitate expanding the storage of feed and the resistance of low pH in the stomach [19–21].

The proliferating *B. subtilis* in the intestine help to maintain an anaerobic environment and lower pH in GIT, which in turn promotes lactic acid and regulates intestinal microflora. It can prevent and minimize GIT diseases [22]. Also, *B. licheniformis* can produce bacitracin, which is against pathogenic microorganisms [23,24]. Bacitracin is a cyclic dodecyl-peptide antibiotic synthesized non-ribosomally by *B. licheniformis* [25]. Bacitracin is directed primarily against gram-positive bacteria via inhibition of the cell wall [26]. Both *B. subtilis* and *B. licheniformis* increased fecal *Lactobacillus* counts in finishing pigs without affecting fecal coliform counts [27]. Additionally, *B. licheniformis* and *B. subtilis* can survive in fecal after excretion and, they sustainedly degrade organic matters in the fecal [28], which could decrease fecal odor and reduce gas formation such as NH_3 production. When growing pigs were given *Bacillus* direct-fed microbial (DFM), methane and NH_3 emissions were reduced by 40% and 50%, respectively [29]. Thus, the *Bacillus* DFM has positive effects on fermentation and protein utilization in older animals.

Accordingly, it has been well-established that dietary *Bacillus* complex supplementation has beneficial effects on weaned to finishing pigs [30–33]. The 1:1 ratio of *B. licheniformis* and *B. subtilis* in growing-finishing pigs increased digestibility and fecal *Lactobacillus* and reduced fecal NH_3 emission [34]. However, there is limited research on the mixing ratio of *Bacillus* complex in dietary supplementation for pigs. Therefore, this study evaluated the effects of different mixing ratios of *B. licheniformis* and *B. subtilis* on nutrient digestibility, fecal microflora, and odor gas emissions in growing pigs.

MATERIALS AND METHODS

The experimental protocol was approved (CBNUA-1619-21-02) by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea.

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Source of probiotics

Probiotic product (Haedamun, Eumseong, Korea) was a mixture of spray-dried spores of *B. licheniformis* (CCTCC WL-04) and *B. subtilis* (CCTCC WL-22). It was guaranteed to contain at least 3×10^{10} CFU kg^{-1} of *B. licheniformis* and *B. subtilis*, respectively.

Experiment design and housing

A total of four crossbred ([Landrace × Yorkshire] × Duroc) barrows were randomly allotted to four diets over four periods in a 4 × 4 Latin square design. The pigs (average initial body weight [BW] of 41.2 ± 0.7 kg) were individually housed in 1.2 m × 0.7 m × 0.96 m stainless steel metabolism cages in an environmentally controlled room.

Diets and feeding

Diets were prepared to meet the NRC [35] nutritional requirements for pigs. Table 1 shows nutritional contents of the main ingredients used in this experiment. Treatments were as follows: Control (CON, basal diet), CON + 0.2% probiotic complex (L4S6, *B. licheniformis* and *B. subtilis* at a 4:6 ratio), CON + 0.2% probiotic complex (L5S5, *B. licheniformis* and *B. subtilis* at a 5:5 ratio), CON + 0.2% probiotic complex (L6S4, *B. licheniformis* and *B. subtilis* at a 6:4 ratio). The experiment was conducted for four weeks. The daily feed allowance was adjusted by 2.7 times the requirement to maintain digestible energy (DE, 2.7 × 110 kcal of DE/kg $BW^{0.75}$) [35]. The daily

Table 1. Chemical composition of basal diets (%, as-fed basis)

Items	Content
Ingredient	
Corn	61.35
Wheat	7.50
Soybean meal	25.10
Wheat bran	3.00
Monocalcium phosphate	1.00
Limestone	1.00
Vitamin premix[1)]	0.10
Mineral premix[2)]	0.20
L-Lysine-HCl (78%)	0.30
DL-Methionine (50%)	0.10
L-Threonine (89%)	0.20
Salt	0.15
Calculated composition	
ME (kcal/kg)	3,360
Crude protein	18.1
Crude fat	5.4
Crude fiber	4.1
Crude ash	4.9
Calcium	0.8
Total phosphorus	0.6

[1)]Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D_3, 1,103 IU; vitamin E, 44 IU; vitamin K, 4.4 mg; riboflavin, 8.3 mg; niacin, 50 mg; thiamine, 4 mg; D-pantothenic, 29 mg; choline, 166 mg; and vitamin B_{12}, 33 μg.

[2)]Provided per kg of complete diet: copper (as $CuSO_4 \cdot 5H_2O$), 12 mg; zinc (as $ZnSO_4$), 85 mg; manganese (as MnO_2), 8 mg; iodine (as KI), 0.28 mg; and selenium (as $Na_2SeO_3 \cdot 5H_2O$), 0.15 mg.

feed was divided in half and mixed with water in a 1:1 ratio and fed at 8 and 17 o'clock. During the experiment, the pigs were allowed to drink water freely.

Sampling and analysis

Each week, the experiment consisted of six days of adaptation period and one day of collecting urine and feces. Total feces were immediately collected in metabolic cages and packaged in plastic bags and stored at −20℃ for the duration of the experiment. Urine was collected once a day into buckets filled with 50 mL of 6 mol/L HCl under metabolic cages. The total urine collected was weighed and stored at −20℃. Fecal samples were dried in a forced air oven, then crushed on 1 mm screens and completely thawed prior to subsample collection for chemical analysis. The procedures used for the determination of dry matter (DM), and crude protein (CP) digestibility values were in accordance with the methods established by the AOAC [36]. 1 g of fecal samples from each cage were diluted with 9 mL of 1% peptone broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and homogenized. The viability of bacteria in fecal samples was obtained by plating a series of 10-fold dilutions (1% peptone solution) on MacConkey agar plates (Difco Laboratories, Detroit, MI, USA) and *Lactobacilli* medium III agar plates (Medium 638, DSMZ, Braunschweig, Germany). *Lactobacilli* medium III agar plates were incubated anaerobically at 39℃ for 48 h. MacConkey agar plates were incubated at 37℃ for 24 h. The number of *Escherichia coli* or *Lactobacillus* colonies was measured immediately after removing the plate from the incubator.

For the odor gas estimation, 150 g of fresh fecal, 100 g of sawdust, and 50 g of urine were mixed. Mixtures of fecal, urine and sawdust were fermented at room temperature of 35℃ for 72 hours. The odor-causing materials (NH_3 and H_2S) were analyzed every 24 hours for 7 days with a gas detector (GV-110S, Gastec, Ayase, Japan) and tube namely, NH_3 detector tube No. 3L, H_2S detector tube No. 4LL.

Statistical analysis

Data generated in the present experiment were analyzed as a randomized design in a 4 × 4 Latin square arrangement of treatments. Data collected during the study were subjected to analysis of variance (ANOVA) for Completely Randomized Design [37] using General Linear Model Procedure (SAS, SAS Institute, Cary, NC, USA). The statistical model used to test the effects of treatment on nutrient digestibility, fecal microflora, and odor gas emissions are presented as follows: $Yij = \mu + Pi + Eij$. Where: Yij = Observed value of a dependent variable; μ = Overall mean; Pi = Effect of different mixing ratios of *B. licheniformis* and *B. subtilis*; and Eij = Residual error. The differences between means were tested for significance ($p < 0.05$) using the least significant difference (LSD) range test.

RESULTS

Nutrient digestibility

Effects of dietary supplementation with *B. licheniformis* and *B. subtilis* probiotics in different mixing ratios on nutrient digestibility in growing pigs are shown in Table 2. CP digestibility values were greater ($p < 0.05$) for probiotic supplementation treatments than for the CON diet. However, there was no significant difference in CP digestibility and DM digestibility among all treatments.

Fecal microflora

Effects of dietary supplementation with *B. licheniformis* and *B. subtilis* probiotics at different mixing ratios on fecal microflora in growing pigs are shown in Table 3. *E. coli* counts in fecal samples were

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Table 2. Effects of supplementation with *Bacillus licheniformis* and *Bacillus subtilis* probiotics at different mixing ratios on nutrient digestibility in growing pigs

Items	CON	L4S6	L5S5	L6S4	SE	*p*-value
Dry matter	83.82	83.81	84.08	83.79	0.07	0.506
Crude protein	70.75[b]	73.81[a]	73.33[a]	73.81[a]	0.48	0.044

Each value is presented as the mean value of four replicates (1 pig / cage; 4 × 4 latin square).

[a,b]Means in the same row with different superscripts differ significantly ($p < 0.05$).

CON, basal diet; L4S6, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 4:6 ratio); L5S5, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 5:5 ratio); L6S4, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 6:4 ratio).

Table 3. Effects of supplementation with *Bacillus licheniformis* and *Bacillus subtilis* probiotics at different mixing ratios on fecal microflora in growing pigs

Items	CON	L4S6	L5S5	L6S4	SE	*p*-value
Lactobacillus	7.387	7.545	7.528	7.512	0.026	0.164
Escherichia coli	5.885[a]	5.584[b]	5.617[b]	5.498[b]	0.039	0.049

Each value is presented as the mean value of four replicates (1 pig / cage; 4 × 4 latin square).

[a,b]Means in the same row with different superscripts differ significantly ($p < 0.05$).

CON, basal diet; L4S6, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 4:6 ratio); L5S5, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 5:5 ratio); L6S4, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 6:4 ratio).

lower ($p < 0.05$) at the probiotic supplementation treatments than the CON diet. However, there was no significant difference in *E. coli* counts between the different mixing ratios of *B. licheniformis* and *B. subtilis*. All treatments did not show a significant difference in *Lactobacillus* counts.

Odor gas emissions

Effects of dietary supplementation with *B. licheniformis* and *B. subtilis* probiotics at different mixing ratios on odor gas emissions in growing pigs are shown in Table 4. There was no significant difference in NH_3 or H_2S emission until day 3. On days 4 and 5, the L4S6 and L5S5 showed lower ($p < 0.05$) fecal H_2S emissions than the CON diet. On day 6, the L6S4 also showed lower H_2S emission ($p < 0.05$). NH_3 emission in the L4S6 and L5S5 was significantly decreased ($p < 0.05$) compared to that in the CON diet. On day 7, all of probiotics supplementation decreased ($p < 0.05$) both NH_3 and H_2S emissions than the CON treatment.

DISCUSSION

Nutrient digestibility

Dietary supplementation with *B. subtilis* and *B. licheniformis* can increase nutrient digestibility by producing extracellular enzymes including proteases and α-amylase [38,39] to improve feed conversion in pigs [40]. In our study, all ratios of *B. licheniformis* and *B. subtilis* complex increased the apparent total tract digestibility (ATTD) of CP compared to the CON diet, but there was no different ATTD of CP among the groups. And all treatments did not improve DM. Many studies argue the advantage of *B. licheniformis* and *B. subtilis* complex in growing and/or finishing pig. The dietary supplementations with *B. subtilis* and *B. licheniformis* were expected more effective in weanling pigs than in growing pigs where they are under the development or impairment of gut microbiota [41]. Similar to our results, the study of Mun et al. [42] showed a tendency to improve digestibility of CP with supplementation of *B. licheniformis* and *B. subtilis* complex in weanling pigs. Lee et al. [30] also reported the positive effects on ATTD of DM and CP in weanling pigs. Nevertheless, Conversely, Chen et al. [43] have reported that *B. subtilis* based multi probiotics show

Table 4. Effects of supplementation with *Bacillus licheniformis* and *Bacillus subtilis* probiotics at different mixing ratios on odor gas emissions in growing pigs

Items	CON	L4S6	L5S5	L6S4	SE	*p*-value
day 1						
Ammonia	11.5	10.8	11.1	11.1	0.1	0.486
Hydrogen sulfide	22.6	21.5	21.8	21.9	0.2	0.417
day 2						
Ammonia	18.4	17.1	16.5	17.8	0.3	0.571
Hydrogen sulfide	24.3	24	24.1	23.7	0.1	0.631
day 3						
Ammonia	26.3	27.4	27.1	25.1	0.5	0.432
Hydrogen sulfide	27.5	25.1	25.7	24.8	0.4	0.185
day 4						
Ammonia	30.2	29.1	28.7	29.5	1.0	0.091
Hydrogen sulfide	38.7^{a}	32.5^{b}	34.8^{b}	35.1^{ab}	0.9	0.049
day 5						
Ammonia	41.5	37.4	38.5	38.6	1.1	0.078
Hydrogen sulfide	50.1^{a}	41.9^{b}	43.4^{b}	46.7^{ab}	1.5	0.032
day 6						
Ammonia	71.5^{a}	60.1^{b}	63.4^{b}	68.9^{ab}	2.8	0.041
Hydrogen sulfide	60.7^{a}	55.8^{b}	56.8^{b}	57.4^{b}	1.1	0.025
day 7						
Ammonia	131.5^{a}	71.4^{b}	80.5^{b}	86.8^{b}	3.4	0.010
Hydrogen sulfide	81.7^{a}	63.3^{b}	67.5^{b}	70.1^{b}	1.9	< 0.001

Each value is presented as the mean value of four replicates (1 pig / cage; 4 × 4 latin square).

^{a,b}Means in the same row with different superscripts differ significantly ($p < 0.05$).

CON, basal diet; L4S6, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 4:6 ratio); L5S5, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 5:5 ratio); L6S4, CON + 0.2% probiotic complex (*B. licheniformis* and *B. subtilis* at a 6:4 ratio).

no effect on ATTD of DM or CP in growing-finishing pigs. On the other hand, a previous study reported dietary supplementation of *B. subtilis* and *B. licheniformis* complex (1:1 ratio) can improve ATTD of DM and CP in growing pigs [33]. Currently, it is difficult to confirm the benefits of *B. subtilis* and *B. licheniformis* complex due to various environmental conditions such as gender, health status, environment, composition of ingredients, and strain of probiotic. Therefore, more research is needed to clarify this.

Fecal microflora

Bacillus-based probiotics have been used steadily due to their positive effects on gut health, such as kinetics of nutrient transport through enterocytes [44], intestinal cell proliferation [45], and modulation of the gut immune system [46,47]. In our study, dietary supplementation with *B. licheniformis* and *B. subtilis* complex in all ratios decreased fecal *E. coli* count, but no difference in fecal *Lactobacillus* count. In general, *Bacillus* supplementation provides more positive and consistent results in weanling pigs than in growing-finishing pigs [48]. During the weaning period, without affecting *Lactobacillus* counts dietary *B. subtilis* can result in fewer coliform counts [30] and the mixture of *B. subtilis* and *B. licheniformis* reduced *E. coli* counts [49]. In contrast, dietary *Bacillus*-based probiotic supplementation in growing-finishing pigs did not indicate the positive effects on the reduction of fecal *Lactobacillus* or *E. coli* counts [50]. This is probably due to the higher

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resistance to intestinal pathogens in growing-finishing pigs.

Odor gas emissions

NH_3 and sulfur-containing compounds are the two most important toxic gases that cause odors and pollute the environment [51]. Exposure to high levels of malodorous gases such as NH_3, volatile sulfur, and volatile organic compounds is not only negatively affecting animal health and performance, but also affects human health and causes environmental problems [52]. The malodorous gases can be reduced by the improvement of nutrient digestibility and gut microbiota composition [53]. Previous studies reported *Bacillus*-based probiotics decrease NH_3 emissions in growing pigs [15,54] and NH_3 and H_2S emissions in sows [55]. As expected, at the end of experiment, NH_3 and H_2S emissions were significantly reduced in all ratios but, the treatments L4S6 and L5S5 showed a significant decrease in H_2S emissions from day 4 and the lower NH_3 from day 6. In our digestibility study, all ratios of *B. licheniformis* and *B. subtilis* complex indicated the increase of ATTD of CP and, the higher digestibility of CP could decrease the fecal NH_3 and H_2S. Improvements in ATTD in CP may reflect decreased NH_3 excretion due to increased protein absorption in the upper GIT and decreased protein fermentation in the lower GIT. The low nitrogen concentration in fecal samples can reduce fecal NH_3 and H_2S emissions [56]. Particularly, *B. subtilis* consumes oxygen in the digestive tract and additionally produces certain enzymes such as subtilisin and catalase that can improve nutrient digestibility [57]. Besides, *B. subtilis* can produce a glycosyl hydrolase, which aids in the hydrolysis of glycosidic bonds in complex sugars [58]. Subsequently, higher *B. subtilis* in the mixtures could accelerate the protein and carbohydrate degradation and reduced the malodorous gases.

CONCLUSION

This study suggested that dietary probiotic supplementation with *B. licheniformis* and *B. subtilis* in growing pigs significantly improved crude protein digestibility. It also significantly reduced *E. coli* counts and gas emission. Although there were no significant differences among different mixing ratios of *B. licheniformis* and *B. subtilis* in digestibility and fecal microflora, odor gas emissions showed significantly different. Particularly, H_2S and NH_3 emissions were decreased in L4S6 and L5S5 treatments. Therefore, the 5:5 or 4:6 ratio of probiotics mixture with *B. licheniformis* and *B. subtilis* has the potential advantage of odor gas reduction.

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