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 NH₃ or H₂S emission until day 3. The positive effect of H₂S and NH₃ 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 H₃S and NH₃ emission in days 4 and 6 (*p* < 0.05). On day 7, all probiotic complexes decreased (*p* < 0.05) H₂S and NH₃ 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, NH₃ and H₂S 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
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 NH3 production. When growing pigs were given Bacillus direct-fed microbial (DFM), methane and NH3 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 NH3 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.

the original work is properly cited.

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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
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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 $3 \times 10^{10}$ 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, $2.7 \times 110$ kcal of DE/kg BW$^{0.75}$) [35].

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

| Ingredient             | Content |  |
|------------------------|---------|---|
| 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$_4$·5H$_2$O), 12 mg; zinc (as ZnSO$_4$), 85 mg; manganese (as MnO$_2$), 8 mg; iodine (as KI), 0.28 mg; and selenium (as Na$_2$SeO$_3$·5H$_2$O), 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°C 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°C. 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°C for 48 h. MacConkey agar plates were incubated at 37°C 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°C for 72 hours. The odor-causing materials (NH₃ and H₂S) were analyzed every 24 hours for 7 days with a gas detector (GV-110S, Gastec, Ayase, Japan) and tube namely, NH₃ detector tube No. 3L, H₂S 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: Yi = μ + Pi + Eij. Where: Yi = 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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lower \( (p < 0.05) \) at the probiotic supplementation treatments than the CON diet. However, there was no significant difference in \textit{E. coli} counts between the different mixing ratios of \textit{B. licheniformis} and \textit{B. subtilis}. All treatments did not show a significant difference in \textit{Lactobacillus} counts.

**Odor gas emissions**

Effects of dietary supplementation with \textit{B. licheniformis} and \textit{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 \( \text{NH}_3 \) or \( \text{H}_2\text{S} \) emission until day 3. On days 4 and 5, the L4S6 and L5S5 showed lower \( (p < 0.05) \) fecal \( \text{H}_2\text{S} \) emissions than the CON diet. On day 6, the L6S4 also showed lower \( \text{H}_2\text{S} \) emission \( (p < 0.05) \). \( \text{NH}_3 \) 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 \( \text{NH}_3 \) and \( \text{H}_2\text{S} \) emissions than the CON treatment.

**DISCUSSION**

**Nutrient digestibility**

Dietary supplementation with \textit{B. subtilis} and \textit{B. licheniformis} can increase nutrient digestibility by producing extracellular enzymes including proteases and \( \alpha \)-amylase \[38,39\] to improve feed conversion in pigs \[40\]. In our study, all ratios of \textit{B. licheniformis} and \textit{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 \textit{B. licheniformis} and \textit{B. subtilis} complex in growing and/or finishing pigs. The dietary suplementations with \textit{B. subtilis} and \textit{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 \textit{B. licheniformis} and \textit{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 \textit{B. subtilis} based multi probiotics show

Table 2. Effects of supplementation with \textit{Bacillus licheniformis} and \textit{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\( ^a \) | 73.81\( ^* \) | 73.33\( ^* \) | 73.81\( ^* \) | 0.48 | 0.044 |

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

\( ^a \)Means in the same row with different superscripts differ significantly \( (p < 0.05) \).

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

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

| Items            | CON | L4S6 | L5S5 | L6S4 | SE | \( p \)-value |
|------------------|-----|------|------|------|----|--------------|
| \textit{Lactobacillus} | 7.387 | 7.545 | 7.528 | 7.512 | 0.026 | 0.164 |
| \textit{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 \( \times \) 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 (\textit{B. licheniformis} and \textit{B. subtilis} at a 4:6 ratio); L5S5, CON + 0.2% probiotic complex (\textit{B. licheniformis} and \textit{B. subtilis} at a 5:5 ratio); L6S4, CON + 0.2% probiotic complex (\textit{B. licheniformis} and \textit{B. subtilis} at a 6:4 ratio).
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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

### 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 | 32.5 | 34.8 | 35.4 | 0.9 | 0.049   |
| day 5              |      |      |      |      |     |         |
| Ammonia            | 41.5 | 37.4 | 38.5 | 38.6 | 1.1 | 0.078   |
| Hydrogen sulfide   | 50.1 | 41.9 | 43.4 | 46.7 | 1.5 | 0.032   |
| day 6              |      |      |      |      |     |         |
| Ammonia            | 71.5 | 60.1 | 63.4 | 68.9 | 2.8 | 0.041   |
| Hydrogen sulfide   | 60.7 | 55.8 | 56.8 | 57.4 | 1.1 | 0.025   |
| day 7              |      |      |      |      |     |         |
| Ammonia            | 131.5| 71.4 | 80.5 | 86.8 | 3.4 | 0.010   |
| Hydrogen sulfide   | 81.7 | 63.3 | 67.5 | 70.1 | 1.9 | < 0.001 |

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

*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).
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$_2$S emissions in sows [55]. As expected, at the end of experiment, NH$_3$ and H$_2$S emissions were significantly reduced in all ratios but, the treatments L4S6 and L5S5 showed a significant decrease in H$_2$S 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 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$_2$S 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$_2$S 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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