

# 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<sub>3</sub> or H<sub>2</sub>S emission until day 3. The positive effect of H<sub>2</sub>S and NH<sub>3</sub> 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<sub>2</sub>S and NH<sub>3</sub> emission in days 4 and 6 ( $p < 0.05$ ). On day 7, all probiotic complexes decreased ( $p < 0.05$ ) H<sub>2</sub>S and NH<sub>3</sub> 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<sub>3</sub> and H<sub>2</sub>S 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<sub>3</sub> production. When growing pigs were given *Bacillus* direct-fed microbial (DFM), methane and NH<sub>3</sub> 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<sub>3</sub> 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.

### 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<sup>-1</sup> 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<sup>0.75</sup>) [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 <sup>1</sup>	0.10
Mineral premix <sup>2</sup>	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

<sup>1</sup>Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D<sub>3</sub>, 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<sub>12</sub>, 33 µg.

<sup>2</sup>Provided per kg of complete diet: copper (as CuSO<sub>4</sub> · 5H<sub>2</sub>O), 12 mg; zinc (as ZnSO<sub>4</sub>), 85 mg; manganese (as MnO<sub>2</sub>), 8 mg; iodine (as KI), 0.28 mg; and selenium (as Na<sub>2</sub>SeO<sub>3</sub> · 5H<sub>2</sub>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  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$ . 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  $39^{\circ}\text{C}$  for 48 h. MacConkey agar plates were incubated at  $37^{\circ}\text{C}$  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  $35^{\circ}\text{C}$  for 72 hours. The odor-causing materials ( $\text{NH}_3$  and  $\text{H}_2\text{S}$ ) were analyzed every 24 hours for 7 days with a gas detector (GV-110S, Gastec, Ayase, Japan) and tube namely,  $\text{NH}_3$  detector tube No. 3L,  $\text{H}_2\text{S}$  detector tube No. 4LL.

### Statistical analysis

Data generated in the present experiment were analyzed as a randomized design in a  $4 \times 4$  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:  $Y_{ij} = \mu + P_i + E_{ij}$ . Where:  $Y_{ij}$  = Observed value of a dependent variable;  $\mu$  = Overall mean;  $P_i$  = Effect of different mixing ratios of *B. licheniformis* and *B. subtilis*; and  $E_{ij}$  = 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

**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 <sup>b</sup>	73.81 <sup>a</sup>	73.33 <sup>a</sup>	73.81 <sup>a</sup>	0.48	0.044

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

<sup>a,b</sup>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
<i>Lactobacillus</i>	7.387	7.545	7.528	7.512	0.026	0.164
<i>Escherichia coli</i>	5.885 <sup>a</sup>	5.584 <sup>b</sup>	5.617 <sup>b</sup>	5.498 <sup>b</sup>	0.039	0.049

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

<sup>a,b</sup>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<sub>3</sub> or H<sub>2</sub>S emission until day 3. On days 4 and 5, the L4S6 and L5S5 showed lower ( $p < 0.05$ ) fecal H<sub>2</sub>S emissions than the CON diet. On day 6, the L6S4 also showed lower H<sub>2</sub>S emission ( $p < 0.05$ ). NH<sub>3</sub> 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<sub>3</sub> and H<sub>2</sub>S 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  $\alpha$ -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 <sup>a</sup>	32.5 <sup>b</sup>	34.8 <sup>b</sup>	35.1 <sup>ab</sup>	0.9	0.049
day 5						
Ammonia	41.5	37.4	38.5	38.6	1.1	0.078
Hydrogen sulfide	50.1 <sup>a</sup>	41.9 <sup>b</sup>	43.4 <sup>b</sup>	46.7 <sup>ab</sup>	1.5	0.032
day 6						
Ammonia	71.5 <sup>a</sup>	60.1 <sup>b</sup>	63.4 <sup>b</sup>	68.9 <sup>ab</sup>	2.8	0.041
Hydrogen sulfide	60.7 <sup>a</sup>	55.8 <sup>b</sup>	56.8 <sup>b</sup>	57.4 <sup>b</sup>	1.1	0.025
day 7						
Ammonia	131.5 <sup>a</sup>	71.4 <sup>b</sup>	80.5 <sup>b</sup>	86.8 <sup>b</sup>	3.4	0.010
Hydrogen sulfide	81.7 <sup>a</sup>	63.3 <sup>b</sup>	67.5 <sup>b</sup>	70.1 <sup>b</sup>	1.9	< 0.001

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

<sup>a,b</sup>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 weaning 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

resistance to intestinal pathogens in growing-finishing pigs.

### Odor gas emissions

NH<sub>3</sub> 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<sub>3</sub>, 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<sub>3</sub> emissions in growing pigs [15,54] and NH<sub>3</sub> and H<sub>2</sub>S emissions in sows [55]. As expected, at the end of experiment, NH<sub>3</sub> and H<sub>2</sub>S emissions were significantly reduced in all ratios but, the treatments L4S6 and L5S5 showed a significant decrease in H<sub>2</sub>S emissions from day 4 and the lower NH<sub>3</sub> 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<sub>3</sub> and H<sub>2</sub>S. Improvements in ATTD in CP may reflect decreased NH<sub>3</sub> 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<sub>3</sub> and H<sub>2</sub>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<sub>2</sub>S and NH<sub>3</sub> 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.

## REFERENCES

1. Chu GM, Lee SJ, Jeong HS, Lee SS. Efficacy of probiotics from anaerobic microflora with prebiotics on growth performance and noxious gas emission in growing pigs. *Anim Sci J*. 2011;82:282-90. <https://doi.org/10.1111/j.1740-0929.2010.00828.x>
2. Flynn D. South Korea bans antibiotics in animal feed [Internet]. *Food Safety News*. 2011 [cited 2022 Jan 7]. <https://www.foodsafetynews.com/2011/06/south-korea-bans-antibiotics-in-animal-feed/>
3. Chu GM, Jung CK, Kim HY, Ha JH, Kim JH, Jung MS, et al. Effects of bamboo charcoal and bamboo vinegar as antibiotic alternatives on growth performance, immune responses and fecal microflora population in fattening pigs. *Anim Sci J*. 2013;84:113-20. <https://doi.org/10.1111/j.1740-0929.2012.01045.x>
4. Martin MJ, Thottathil SE, Newman TB. Antibiotics overuse in animal agriculture: a call to action for health care providers. *Am J Public Health*. 2015;105:2409-10. <https://doi.org/10.1111/ajph.12700>

- org/10.2105/AJPH.2015.302870
5. Walsh TR, Wu Y. China bans colistin as a feed additive for animals. *Lancet Infect Dis.* 2016;16:1102-3. [https://doi.org/10.1016/S1473-3099\(16\)30329-2](https://doi.org/10.1016/S1473-3099(16)30329-2)
  6. Jongbloed AW, Lenis NP. Alteration of nutrition as a means to reduce environmental pollution by pigs. *Livest Prod Sci.* 1992;31:75-94. [https://doi.org/10.1016/0301-6226\(92\)90057-B](https://doi.org/10.1016/0301-6226(92)90057-B)
  7. Paik IK, Blair R, Jacob J. Strategies to reduce environmental pollution from animal manure: principles and nutritional management - a review. *Asian-Australas J Anim Sci.* 1996;9:615-36. <https://doi.org/10.5713/ajas.1996.615>
  8. Jeong TS, Heo PS, Lee GY, Kim DH, Ju WS, Kim YY. The influence of phase feeding methods on growth performance, meat quality, and production cost in growing-finishing pigs. *J Anim Sci Technol.* 2010;52:29-36. <https://doi.org/10.5187/JAST.2010.52.1.029>
  9. Nousiainen J. Comparative observations on selected probiotics and olaquinox as feed additives for piglets around weaning: 2. effect on villus length and crypt depth in the jejunum, ileum, caecum and colon. *J Anim Physiol Anim Nutr.* 1991;66:224-30. <https://doi.org/10.1111/j.1439-0396.1991.tb00290.x>
  10. Davila AM, Blachier F, Gotteland M, Andriamihaja M, Benetti PH, Sanz Y, et al. Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. *Pharmacol Res.* 2012;68:95-107. <https://doi.org/10.1016/j.phrs.2012.11.005>
  11. Jha R, Berrocso JFD. Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: a review. *Anim Feed Sci Technol.* 2016;212:18-26. <https://doi.org/10.1016/j.anifeedsci.2015.12.002>
  12. Simon O. Micro-organisms as feed additives-probiotics. *Adv Pork Prod.* 2005;16:161-7.
  13. Le Bon M, Davies HE, Glynn C, Thompson C, Madden M, Wiseman J, et al. Influence of probiotics on gut health in the weaned pig. *Livest Sci.* 2010;133:179-81. <https://doi.org/10.1016/j.livsci.2010.06.058>
  14. Zimmermann JA, Fusari ML, Rossler E, Blajman JE, Romero-Scharpen A, Astesana DM, et al. Effects of probiotics in swines growth performance: a meta-analysis of randomised controlled trials. *Anim Feed Sci Technol.* 2016;219:280-93. <https://doi.org/10.1016/j.anifeedsci.2016.06.021>
  15. Payling L, Kim IH, Walsh MC, Kiarie E. Effects of a multi-strain *Bacillus* spp. direct-fed microbial and a protease enzyme on growth performance, nutrient digestibility, blood characteristics, fecal microbiota, and noxious gas emissions of grower pigs fed corn-soybean-meal-based diets—a meta-analysis. *J Anim Sci.* 2017;95:4018-29. <https://doi.org/10.2527/jas.2017.1522>
  16. Gaggia F, Mattarelli P, Biavati B. Probiotics and prebiotics in animal feeding for safe food production. *Int J Food Microbiol.* 2010;141:S15-28. <https://doi.org/10.1016/j.ijfoodmicro.2010.02.031>
  17. Kang J, Lee JJ, Cho JH, Choe J, Kyoung H, Kim SH, et al. Effects of dietary inactivated probiotics on growth performance and immune responses of weaned pigs. *J Anim Sci Technol.* 2021;63:520-30. <https://doi.org/10.5187/jast.2021.e44>
  18. Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, et al. Non-antibiotic feed additives in diets for pigs: a review. *Anim Nutr.* 2018;4:113-25. <https://doi.org/10.1016/j.aninu.2018.01.007>
  19. Feliciano MAR, Saad FMOB, Logato PVR, Aquino AA, José VA, Roque NC. Effects of probiotics on digestibility, faecal score, and hematologic characteristics in dogs. *Arq Bras Med Vet Zootec.* 2009;61:1268-74. <https://doi.org/10.1590/S0102-09352009000600003>
  20. Félix AP, Netto MVT, Murakami FY, Marcon de Brito C, Gisele de Oliveira S, Maiorka A.



- Digestibilidade e características das fezes de cães suplementados com *Bacillus subtilis* na dieta. *Cienc Rural*. 2010;40:2169-73. <https://doi.org/10.1590/S0103-84782010005000166>
21. Cutting SM. *Bacillus* probiotics. *Food Microbiol*. 2011;28:214-20. <https://doi.org/10.1016/j.fm.2010.03.007>
  22. Hu Y, Dun Y, Li S, Zhao S, Peng N, Liang Y. Effects of *Bacillus subtilis* KN-42 on growth performance, diarrhea and faecal bacterial flora of weaned piglets. *Asian-Australas J Anim Sci*. 2014;27:1131-40. <https://doi.org/10.5713/ajas.2013.13737>
  23. Kayalvizhi N, Gunasekaran P. Production and characterization of a low-molecular-weight bacteriocin from *Bacillus licheniformis* MKU3. *Lett Appl Microbiol*. 2008;47:600-7. <https://doi.org/10.1111/j.1472-765X.2008.02473.x>
  24. Anthony T, Rajesh T, Kayalvizhi N, Gunasekaran P. Influence of medium components and fermentation conditions on the production of bacteriocin(s) by *Bacillus licheniformis* AnBa9. *Bioresour Technol*. 2009;100:872-7. <https://doi.org/10.1016/j.biortech.2008.07.027>
  25. Ishihara H, Takoh M, Nishibayashi R, Sato A. Distribution and variation of bacitracin synthetase gene sequences in laboratory stock strains of *Bacillus licheniformis*. *Curr Microbiol*. 2002;45:18-23. <https://doi.org/10.1007/s00284-001-0041-5>
  26. MacIver RH, Stewart R, Frederiksen JW, Fullerton DA, Horvath KA. Topical application of bacitracin ointment is associated with decreased risk of mediastinitis after median sternotomy. *Heart Surg Forum*. 2006;9:E750-3. <https://doi.org/10.1532/hsf98.20051187>
  27. Kornegay ET, Risley CR. Nutrient digestibilities of a corn-soybean meal diet as influenced by *Bacillus* products fed to finishing swine. *J Anim Sci*. 1996;74:799-805. <https://doi.org/10.2527/1996.744799x>
  28. Vainshtein M. Probiotics for environmental sanitation: goals and examples. In: Cao G, Orrù R, editors. *Current environmental issues and challenges*. Dordrecht: Springer; 2014. p. 127-35.
  29. Prenafeta-Boldú FX, Fernández B, Viñas M, Lizardo R, Brufau J, Owusu-Asiedu A, et al. Effect of *Bacillus* spp. direct-fed microbial on slurry characteristics and gaseous emissions in growing pigs fed with high fibre-based diets. *Animal*. 2017;11:209-18. <https://doi.org/10.1017/S1751731116001415>
  30. Lee SH, Ingale SL, Kim JS, Kim KH, Lokhande A, Kim EK, et al. Effects of dietary supplementation with *Bacillus subtilis* LS 1-2 fermentation biomass on growth performance, nutrient digestibility, cecal microbiota and intestinal morphology of weanling pig. *Anim Feed Sci Technol*. 2014;188:102-10. <https://doi.org/10.1016/j.anifeedsci.2013.12.001>
  31. FEEDAP [EFSA Panel on Additives and Products or Substances used in Animal Feed]. Safety and efficacy of BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) as a feed additive for sows, piglets, pigs for fattening, turkeys for fattening and calves. *EFSA J*. 2016;14:e04558. <https://doi.org/10.2903/j.efsa.2016.4558>
  32. Balasubramanian B, Li T, Kim IH. Effects of supplementing growing-finishing pig diets with *Bacillus* spp. probiotic on growth performance and meat-carcass grade quality traits. *Rev Bras Zootec*. 2016;45:93-100. <https://doi.org/10.1590/S1806-92902016000300002>
  33. Lewton JR, Woodward AD, Moser RL, Thelen KM, Moeser AJ, Trottier NL, et al. Effects of a multi-strain *Bacillus subtilis*-based direct-fed microbial on weanling pig growth performance and nutrient digestibility. *Transl Anim Sci*. 2021;5:txab058. <https://doi.org/10.1093/tas/txab058>
  34. Lan R, Kim IH. Effects of *Bacillus licheniformis* and *Bacillus subtilis* complex on growth performance and faecal noxious gas emissions in growing-finishing pigs. *J Sci Food Agric*. 2019;99:1554-60. <https://doi.org/10.1002/jsfa.9333>
  35. NRC [National Research Council]. *Nutrient requirements of swine*. 10th rev. ed. Washington,

- DC: National Academies Press; 1998.
36. AOAC [Association of Official Analytical Chemists] International. Official methods of analysis of AOAC International. 16th ed. Washington, DC: AOAC International; 1995.
  37. Steel RGD, Torrie JH. Principles and procedures of statistics: a biometrical approach. 2nd ed. New York, NY: McGraw-Hill; 1980.
  38. Priest FG. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol Rev*. 1977;41:711-53. <https://doi.org/10.1128/br.41.3.711-753.1977>
  39. Carlisle GE, Falkinham JO 3rd. Enzyme activities and antibiotic susceptibility of colonial variants of *Bacillus subtilis* and *Bacillus licheniformis*. *Appl Environ Microbiol*. 1989;55:3026-8. <https://doi.org/10.1128/aem.55.11.3026-3028.1989>
  40. Davis ME, Parrott T, Brown DC, de Rodas BZ, Johnson ZB, Maxwell CV, et al. Effect of a *Bacillus*-based direct-fed microbial feed supplement on growth performance and pen cleaning characteristics of growing-finishing pigs. *J Anim Sci*. 2008;86:1459-67. <https://doi.org/10.2527/jas.2007-0603>
  41. Stavric S, Kornegay ET. Microbial probiotic for pigs and poultry. In: Wallace RJ, Chesson A, editors. *Biotechnology in animal feeds and animal feeding*. Weinheim: Wiley-VCH; 1995. p. 205-31.
  42. Mun D, Kyoung H, Kong M, Ryu S, Jang KB, Baek J, et al. Effects of *Bacillus*-based probiotics on growth performance, nutrient digestibility, and intestinal health of weaned pigs. *J Anim Sci Technol*. 2021;63:1314-27. <https://doi.org/10.5187/jast.2021.e109>
  43. Chen YJ, Min BJ, Cho JH, Kwon OS, Son KS, Kim HJ, et al. Effects of dietary *Bacillus*-based probiotic on growth performance, nutrients digestibility, blood characteristics and fecal noxious gas content in finishing pigs. *Asian-Australas J Anim Sci*. 2006;19:587-92. <https://doi.org/10.5713/ajas.2006.587>
  44. Walsh MC, Rostagno MH, Gardiner GE, Sutton AL, Richert BT, Radcliffe JS. Controlling *Salmonella* infection in weanling pigs through water delivery of direct-fed microbials or organic acids. Part I: effects on growth performance, microbial populations, and immune status. *J Anim Sci*. 2012;90:261-71. <https://doi.org/10.2527/jas.2010-3598>
  45. Babińska I, Rotkiewicz T, Otrocka-Domagala I. The effect of *Lactobacillus acidophilus* and *Bifidobacterium* spp. administration on the morphology of the gastrointestinal tract, liver and pancreas in piglets. *Pol J Vet Sci*. 2005;8:29-35.
  46. Scharek L, Guth J, Filter M, Schmidt MFG. Impact of the probiotic bacteria *Enterococcus faecium* NCIMB 10415 (SF68) and *Bacillus cereus* var. *toyoi* NCIMB 40112 on the development of serum IgG and faecal IgA of sows and their piglets. *Arch Anim Nutr*. 2007;61:223-34. <https://doi.org/10.1080/17450390701431540>
  47. Schierack P, Wieler LH, Taras D, Herwig V, Tachu B, Hlinak A, et al. *Bacillus cereus* var. *toyoi* enhanced systemic immune response in piglets. *Vet Immunol Immunopathol*. 2007;118:1-11. <https://doi.org/10.1016/j.vetimm.2007.03.006>
  48. Mingmongkolchai S, Panbangred W. *Bacillus* probiotics: an alternative to antibiotics for livestock production. *J Appl Microbiol*. 2018;124:1334-46. <https://doi.org/10.1111/jam.13690>
  49. Hu J, Kim YH, Kim IH. Effects of two *Bacillus* strains probiotic supplement on reproduction performance, nutrient digestibility, blood profile, fecal score, excreta odor contents and fecal microflora in lactation sows, and growth performance in sucking piglets. *Livest Sci*. 2021;244:104293. <https://doi.org/10.1016/j.livsci.2020.104293>
  50. Giang HH, Viet TQ, Ogle B, Lindberg JE. Effects of supplementation of probiotics on the performance, nutrient digestibility and faecal microflora in growing-finishing pigs. *Asian-Australas J Anim Sci*. 2011;24:655-61. <https://doi.org/10.5713/ajas.2011.10238>

51. Jongbloed AW, Lenis MP. Excretion of nitrogen and some minerals by livestock. In: Nitrogen flow in pig production and environmental consequences: proceedings of the First International Symposium on Nitrogen Flow in Pig Production and Environmental Consequences; 1993; Wageningen, Netherlands. p. 22-36.
52. Ushida K, Hashizume K, Miyazaki K, Kojima Y, Takakuwa S. Isolation of *Bacillus* sp. as a volatile sulfur-degrading bacterium and its application to reduce the fecal odor of pig. *Asian-Australas J Anim Sci.* 2003;16:1795-8. <https://doi.org/10.5713/ajas.2003.1795>
53. Ferket PR, van Heugten E, van Kempen TATG, Angel R. Nutritional strategies to reduce environmental emissions from nonruminants. *J Anim Sci.* 2002;80:E168-82. [https://doi.org/10.2527/animalsci2002.80E-Suppl\\_2E168x](https://doi.org/10.2527/animalsci2002.80E-Suppl_2E168x)
54. Upadhaya SD, Kim SC, Valientes RA, Kim IH. The effect of *Bacillus*-based feed additive on growth performance, nutrient digestibility, fecal gas emission, and pen cleanup characteristics of growing-finishing pigs. *Asian-Australas J Anim Sci.* 2015;28:999-1005. <https://doi.org/10.5713/ajas.15.0066>
55. Jeong J, Kim J, Lee S, Kim I. Evaluation of *Bacillus subtilis* and *Lactobacillus acidophilus* probiotic supplementation on reproductive performance and noxious gas emission in sows. *Ann Anim Sci.* 2015;15:699-710. <https://doi.org/10.1515/aoas-2015-0018>
56. Cho JH, Chen YJ, Min BJ, Yoo JS, Wang Y, Kim IH. Effects of reducing dietary crude protein on growth performance, odor gas emission from manure and blood urea nitrogen and IGF-1 concentrations of serum in nursery pigs. *Anim Sci J.* 2008;79:453-9. <https://doi.org/10.1111/j.1740-0929.2008.00549.x>
57. Kampf D. Mode of action of *Bacillus subtilis* and efficiency in piglet feeding. *Feed Compounder*; 2012;2:36-7.
58. Larsen N, Thorsen L, Kpikpi EN, Stuer-Lauridsen B, Cantor MD, Nielsen B, et al. Characterization of *Bacillus* spp. strains for use as probiotic additives in pig feed. *Appl Microbiol Biotechnol.* 2014;98:1105-18. <https://doi.org/10.1007/s00253-013-5343-6>