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Research article

Effects of *Bacillus subtilis* C-3102 on growth performance, nutrient digestibility, ammonia gas emission and fecal microbiota of grower-finisher pigs

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Abstract

Importance of the work: *Bacillus subtilis* C-3102 can be used as a probiotic feed additive to improve the production performance of grower-finisher pigs.

Objectives: To evaluate the effects of *B. subtilis* C-3102 on the growth performance, nutrient digestibility and fecal microbiota of grower-finisher pigs.

Materials & Methods: In total, 72 crossbred pigs (mean weight ± SD; 30.73 ± 3.24 kg) were allocated to two groups. Each group received basal feed with or without *B. subtilis* C-3102 ($3^{\cdot\cdot}\times10^5$ colony forming units/g feed) over the feeding period.

Results: The final body weight ($p < 0.05$) and average daily gain ($p < 0.05$) were higher and the feed conversion ratio ($p < 0.05$) was lower in the probiotic group than the control. Dry matter digestibility was higher in the probiotic group ($p < 0.05$) at the end of the grower period. Lower slurry pH ($p < 0.05$) was observed in the probiotic group. Fecal bacterial culture showed that with time, *Escherichia coli* and *Clostridium perfringens* numbers increased ($p < 0.05$) and that the probiotic group had lower *E. coli* numbers than the control group throughout the study ($p < 0.05$). Molecular-based analysis further elucidated different fecal microbial compositions between the groups ($p < 0.05$) and that the microbiota of the finisher pigs were more diverse than for the grower pigs ($p < 0.05$).

Main finding: The supplementation of *B. subtilis* C-3102 in feed was effective in improving the growth performance of grower-finisher pigs and the modification of gut microbiota was likely to be one of the mechanisms of such improvement.

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Introduction

Probiotics are defined as live microbial food supplements which beneficially affect the host animal by improving intestinal microbial balance (Fuller, 1989). Today, they are widely used in animal production (Bhogoju and Nahashon, 2022). In the swine sector, probiotics are often used as the alternative for antibiotics in the post-weaning piglets for the purpose of controlling post-weaning diarrhea and growth promotion (Hu et al., 2014; Su et al., 2022). The use of probiotics in grower-finisher pigs has received relatively less attention compared to younger piglets. Nevertheless, studies have shown the positive effects of *Bacillus* probiotics for weight gain, feed efficiency, odor reduction or carcass quality in grower-finisher pigs (Alexopoulos et al., 2004; Giang et al., 2011; Balasubramanian et al., 2016). Some more recent studies using next-generation sequencing have provided further evidence for a link between the gut microbiota and growth performance of grower-finisher pigs (McCormack et al., 2017; Wang et al., 2019; Gardiner et al., 2020).

Bacillus subtilis C-3102 is a commercial probiotic strain used worldwide for more than 30 years (Jeong and Kim, 2014). It has been approved as a feed additive in the European Union (EU) for species including grower-finisher pigs. Its ability to modify gut microbiota has been demonstrated in various animal species including poultry (Jeong and Kim, 2014; Guyard-Nicodème et al., 2016; Fritts et al., 2000; Suganuma et al., 2021) and pigs (Maruta et al., 1996) and dogs (de Lima et al., 2020), as well as in humans (Takimoto et al., 2018). It has reduced fecal odor and ammonia in poultry (Blair et al., 2004) and dogs (Félix et al., 2010; Paap et al., 2016; Schauf et al., 2019; de Lima et al., 2020). However, such effects have not been studied systematically in grower-finisher pigs. The current study was the first to investigate the effects of *B. subtilis* C-3102 on grower-finisher pigs in tropical climates. As the probiotic is an authorized feed additive in the EU, the outcome of this study should be of particular interest to pig producers in tropical countries who export their products to EU member states.

The current we evaluated the effects of *B. subtilis* C-3102 on the gut microbiota, growth performance, nutrient digestibility, slurry pH and ammonia emission of grower-finisher pigs to elucidate possible mechanisms by which the probiotic confers benefits to pig production.

Materials and Methods

This study was approved by the Animal Ethic Committee of the Bangkok Animal Research Center (BARC) under protocol number AS20168B and was conducted following the guidelines for using animals for scientific purpose of the National Research Council of Thailand, under the Thai Act on Animals for Scientific Purposes, BE 2558 (CE 2015).

Source of probiotic

A commercially available probiotic product Calsporin® (Asahi Biocycle Co., Ltd.; Tokyo, Japan) was used, containing *B. subtilis* C-3102 (1×10^{10} colony forming units (CFU)/g) as active ingredient.

Animals, housing and experimental design

In total, 72 crossbred pigs (1/4 Landrace × 1/4 Large White × 1/2 Duroc) with a mean (±SD) weight of 30.73±3.24 kg and obtained from a local farm in Chonburi province, Thailand were blocked by sex and initial body weight and allocated to two treatment groups (3 pigs/pen, 12 pens/treatment). Specifically, the pigs were first grouped by sex. Within each sex category, the pigs were divided into six blocks (6 pigs/block) according to their body weight in a way that pigs of similar weight were grouped together. Then, the pigs within the same block were divided into two pens (3 pigs/pen) which were subsequently assigned with either of the treatments. This process ensured the two treatment groups had identical average pig body weights at the beginning of the study (30.73±3.31 kg for both the control and probiotic groups). The experiment was undertaken in a closed house with tunnel ventilation and an evaporative cooling system. Pigs were housed in concrete-floored pens (1.5 m × 2.0 m) and had free access to their specified feed and water via a tubular feeder and a nipple drinker, respectively. Pigs were fed *ad libitum* with the same basal feed supplemented with or without *B. subtilis* C-3102 (3×10^{5} CFU/g feed; based on product recommendations). Three types of basal feeds for different growing phases (Grower 1 for days 1–19, Grower 2 for days 20–52 and Finisher for days 53–67) were formulated to meet or exceed the estimated nutrient requirements for growing pigs (Rostagno et al., 2017). No antibiotics or pharmacological levels of minerals were included in the feed. All feeds were provided in mash form (Table 1).

Table 1 Basal feed composition

Item	Feeding phase		
	Grower 1 (days 1–19)	Grower 2 (days 20–52)	Finisher (days 53–67)
Ingredient (%)			
Corn	56.89	68.52	77.44
Soybean meal (dehulled; 48% crude protein)	22.06	16.38	11.64
Broken rice	15.00	10.00	5.00
Soybean oil	1.91	1.56	1.92
Monodicalcium phosphate	1.60	1.24	2.30
Limestone	0.76	0.60	0.05
Swine premix (grower)*	0.25	0.25	0.25
Salt	0.45	0.42	0.40
L-Lysine HCl	0.44	0.44	0.44
L-Threonine	0.19	0.17	0.15
DL-Methionine	0.16	0.13	0.10
L-Tryptophan	0.04	0.04	0.05
L-Valine	-	0.01	0.02
Antimold†	0.20	0.20	0.20
Mycotoxin binder‡	0.05	0.05	0.05
Chemical composition (calculated)			
Dry matter (%)	89.38	89.32	89.42
Crude protein (%)	17.01	14.75	12.74
Crude fat (%)	4.64	4.57	5.12
Linoleic acid (%)	2.20	2.21	2.52
Crude fiber (%)	2.08	2.00	1.90
Metabolizable energy (kcal/kg)	3350	3350	3350
Net energy (kcal/kg)	2516	2551	2571

*Vitamin and mineral premix provided per kilogram of diet: vitamin A 8,000 international units (IU), vitamin D3 2,000 IU, vitamin E 10 mg, vitamin K3 2 mg, vitamin B1 1 mg, vitamin B2 4 mg, vitamin B6 1 mg, vitamin B12 15 µg, niacin 15 mg, choline chloride 200 mg, calcium pantothenate 10 mg, cobalt 1 mg, copper 160 mg, ferrous 150 mg, manganese 40 mg, zinc 112 mg, iodine 1 mg and selenium 0.1 mg.
†Propimpex® (Impextraco; Belgium).
‡Mycofix® Plus 5.0 (Biomin; Austria).

Sampling and measurements

Body weight (BW) and feed intake (FI) were measured on a pen by pen basis on days 0, 20, 40 and 68 for the determination of the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) for each period. The levels of apparent fecal digestibility (AFD) of dry matter, gross energy, nitrogen and crude fiber were determined at the end of the second grower phase. For this purpose, fecal samples were collected from each pen on days 51–53 for pigs received diets containing 0.3% chromic oxide from days 47–53 of the experiment. Feed and fecal samples were stored at -20°C until analysis of digestibility. Fresh fecal and urine samples were collected from each pen on days 45 and 66 to investigate the fecal microbiota, slurry pH and ammonia emission.

Nutrient digestibility

Fecal samples from each pen were pooled, homogenized and freeze-dried. Separately each freeze-dried sample, as well as each feed sample, were ground and analyzed for gross energy (Automatic Bomb Calorimeter; Leco AC500; USA), nitrogen (ISO 5983-2), crude fiber (AOAC 978.10), dry matter (AOAC 930.15) and chromic oxide (Bolin, 1952). The AFD was calculated using following Equation 1:

$$\text{AFD (\%)} = \frac{\left(\frac{N_d}{Cr_d} - \frac{N_f}{Cr_f}\right)}{\frac{N_d}{Cr_d}} \times 100 \quad (1)$$

where N_d is the nutrient concentration in the diet, N_f is the nutrient concentration in the feces sample, Cr_d is the chromic oxide concentration in the diet and Cr_f is the chromic oxide concentration in feces sample.

Bacterial culture

A sample (1 g) of fecal sample was homogenized in 9 mL of Mitsuoka's buffer (Mitsuoka et al., 1965; Muto et al., 2010) and 10-fold serial dilutions were made. Diluted samples were spread onto CHROMagar™ E. coli, CHROMagar™ C. perfringens (CHROMagar, France), BBL™ Trypticase™ Soy Broth (BD, USA) with 1.8% agar and BL Agar (Eiken Chemical, Japan) supplemented with 4% sheep blood for determination of viable counts of *Escherichia coli*, *Clostridium perfringens*, *B. subtilis* C-3102 and total anaerobes, respectively. CHROMagar™ C. perfringens and BL Agar were incubated anaerobically at 37°C for 48 hr. Other plates were incubated aerobically at 37°C for 24 hr. Numbers of *B. subtilis* C-3102 were determined by counting the colonies showing the morphology typical of this strain.

Slurry pH and ammonia gas emission

An amount (50 g) each of fresh feces and urine sample collected from the three pigs per pen were mixed and pooled by pen. The pH of each mixture was measured using a pH meter. A sample (100 g) of each mixture was incubated in a closed plastic container for 24 hr at ambient temperature (25°C). The headspace ammonia gas concentration was measured using an MX6 iBrid multi-gas monitor (Industrial Scientific; USA).

DNA extraction, 16S rRNA gene amplification and sequencing

Total DNA was directly extracted from fresh feces according to the method detailed in Takimoto et al. (2018), with minor modifications. The concentration and quality of extracted DNA were analyzed using a spectrophotometer (BioPhotometer® D30; Eppendorf; Germany). The V3–V4 region of the 16S rRNA gene was amplified using the primers 341F (5'-CCTAYGGGRBGCASCAG) and 806R (5'-GGACTACNNGGGTATCTAAT) with barcodes. Polymerase chain reaction (PCR) was carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs; USA). The PCR products were checked based on electrophoresis on 2% agarose gel, mixed at equal density ratios and purified using a Qiagen Gel Extraction Kit (Qiagen; Germany). The libraries were generated with a NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs; USA) before sequencing on an Illumina NovaSeq 6000 platform (Illumina; USA). Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences, then merged using the FLASH software (version 1.2.7), according to Magoč and Salzberg (2011). Quality filtering on the raw tags was performed to obtain high-quality clean tags according to the QIIME (version 1.7.0) quality control process (Caporaso et al., 2010; Bokulich et al., 2013). The tags were compared with the reference database using the UCHIME algorithm to detect chimera sequences. Analysis was performed using the UPARSE software (version 7.0.1001; Edgar, 2003) using all effective tags. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic unit (OTU). Taxonomic annotation was performed by comparing representative sequences from each OTU against the SSUrRNA database of SILVA. Simpson's diversity index calculation and principal component (PC) analysis were performed on the basis of OTU abundance.

Statistical analysis

Growth performance and nutrient digestibility were analyzed based on a randomized complete block design with pen considered as the experimental unit and blocked by sex and initial body weight. Treatment effect was computed based on analysis of variance (ANOVA) using the SAS software (version 9.4; SAS; USA). Fecal microbiota, slurry pH and ammonia gas emission were analyzed based on two-way ANOVA in a factorial arrangement of time and treatment, followed by *post-hoc* Tukey's honest significance test using the Statistix 10 software (Analytical Software; USA). Student's t test was performed for the evaluation of treatment effect at different time points when an interaction of treatment and time was observed.

Results

Growth performance and nutrient digestibility

Inclusion of *B. subtilis* C-3102 did not affect the growth performance for the first 20 d of the study period; however, it did improve BW ($p < 0.05$) and ADG ($p < 0.05$) for days 21–40, days 41–68 and for the whole study period. FCR improved during the days 21–40 ($p = 0.046$) and for the whole study period ($p = 0.026$), as shown in Table 2. Dry matter digestibility was improved in the probiotic group ($p = 0.001$), but no difference was observed in gross energy ($p = 0.352$), nitrogen ($p = 0.605$) and crude fiber ($p = 0.525$), as shown in Table 3.

Table 2 Effects of *Bacillus subtilis* C-3102 on growth performance of grower-finisher pigs

Period	Parameter	T1 Control	T2 Probiotic	SEM	*p* value
Initial BW (kg)		30.73±3.31	30.73±3.31	0.041	0.970
Days 1–20	ADG (kg)	1.007±0.070	1.022±0.056	0.011	0.381
	ADFI (kg)	1.946±0.158	1.968±0.097	0.025	0.540
	FCR	1.932±0.073	1.928±0.060	0.015	0.856
	Final BW (kg)	50.87±4.53	51.16±3.91	0.241	0.407
Days 21–40	ADG (kg)	1.120±0.061	1.170±0.039	0.014	0.024
	ADFI (kg)	2.711±0.180	2.719±0.106	0.039	0.883
	FCR	2.422±0.110	2.322±0.103	0.031	0.046
	Final BW (kg)	73.26±4.28	74.60±4.12	0.284	0.007
Days 41–68	ADG (kg)	1.056±0.042	1.091±0.034	0.010	0.030
	ADFI (kg)	2.769±0.079	2.787±0.128	0.033	0.706
	FCR	2.624±0.101	2.554±0.086	0.036	0.194
	Final BW (kg)	102.84±4.75	105.16±4.36	0.485	0.006
Days 1–68	ADG (kg)	1.060±0.034	1.094±0.036	0.007	0.006
	ADFI (kg)	2.507±0.080	2.522±0.091	0.011	0.339
	FCR	2.365±0.043	2.305±0.055	0.016	0.026
	Final BW (kg)	102.84±4.75	105.16±4.36	0.485	0.006

Each value represents the mean ± SD of 12 replicates. SEM = standard error of the mean; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio

Table 3 Effects of *Bacillus subtilis* C-3102 on digestibility of nutrients (days 51–53)

Item	T1 Control	T2 Probiotic	SEM	*p* value
Dry matter (%)	94.52±0.65	95.55±0.44	0.172	0.001
Gross energy (%)	83.29±1.22	83.71±1.30	0.305	0.352
Nitrogen (%)	78.91±1.64	79.30±2.37	0.518	0.605
Crude fiber (%)	44.83±3.43	45.73±1.99	0.970	0.525

Each value represents the mean ± SD of 12 replicates; SEM = standard error of the mean

Fecal bacterial count, slurry pH and ammonia gas emission

Fecal bacterial culture analysis showed higher numbers of *E. coli* and *C. perfringens* on day 66 than on day 45 ($p < 0.001$), as shown in (Table 4). The probiotic group had lower *E. coli* counts than the control group ($p < 0.001$). Numbers of the probiotic strain, *B. subtilis* C-3102, were higher in the probiotic group ($p < 0.001$). The low levels of *B. subtilis* C-3102 detected in the control group could have been non-probiotic *Bacillus* originating from the feed raw materials which had a similar colony morphology to the probiotic strain. No difference was observed in the total anaerobes between groups ($p = 0.632$). Slurry pH was lower in probiotic group than control group ($p = 0.021$). No difference was detected in the slurry ammonia gas emission between groups ($p > 0.05$).

Table 4 Effects of *Bacillus subtilis* C-3102 on fecal bacterial count, slurry pH and ammonia gas emission

Item	Day 45		Day 66		SEM	*p* value		
	T1 Control	T2 Probiotic	T1 Control	T2 Probiotic		Day	Group	Day × Group
Bacterial count (log_{10}CFU/g)								
E. coli	6.9±0.4	6.1±0.4	7.2±0.5	7.0±0.5	0.19	< 0.001	< 0.001	0.056
C. perfringens	7.2±0.3	7.0±0.3	7.8±0.3	7.8±0.4	0.14	< 0.001	0.257	0.184
B. subtilis C-3102	3.8±0.7	5.8±0.1	4.0±0.2	5.8±0.1	0.15	0.432	< 0.001	0.557
Total anaerobes	9.5±0.4	9.6±0.3	9.5±0.2	9.5±0.3	0.13	0.415	0.632	0.655
pH	5.87±0.23	5.72±0.25	5.84±0.11	5.72±0.15	0.08	0.812	0.021	0.733
Ammonia (ppm)	23.1±8.3	21.7±7.3	25.7±8.6	22.3±8.7	3.37	0.513	0.326	0.685

Each value represents the mean ± SD of 12 replicates; SEM = standard error of the mean; CFU = colony forming unit; ppm = parts per million

Fecal microbiome based on 16S rRNA gene sequencing

The relative abundance levels of different bacterial phyla and genera comprising more than 5% of the fecal microbiota is shown in Table 5. At the phylum level, with time, the Firmicutes decreased ($p < 0.001$) and the Euryarchaeota increased ($p = 0.006$). Interaction of day and group was detected in the Proteobacteria ($p = 0.043$). At the genus level, with time, *Methanobrevibacter* and the Christensenellaceae R-7 group increased ($p < 0.05$) and *Streptococcus* decreased ($p < 0.001$). The interaction of day and group for *Succinivibrio* and *Lactobacillus* prompted evaluation of the treatment effect at each time point. These results indicated that compared to the control group, *Lactobacillus* was lower in the probiotic group on day 45 ($p = 0.048$) but higher on day 66 ($p = 0.048$). Simpson's diversity index was influenced by day as indicated by the higher bacterial diversity on day 66 than day 45 ($p = 0.010$), as shown in Table 5.

Table 5 Effects of *Bacillus subtilis* C-3102 on relative abundance of fecal microbes

Item	Day 45		Day 66		SEM	*p* value		
	T1 Control	T2 Probiotic	T1 Control	T2 Probiotic		Day	Group	Day × Group
Phylum (%)								
Firmicutes	55.1±3.0	55.3±3.5	51.8±3.1	51.9±3.3	1.33	0.001	0.869	0.942
Bacteroidetes	27.9±2.3	27.8±3.2	29.1±2.7	28.9±3.4	1.19	0.163	0.858	0.942
Euryarchaeota	9.2±2.7	7.8±2.0	10.1±2.6	11.1±2.8	1.04	0.006	0.766	0.113
Proteobacteria	5.9±2.4	7.4±1.6	7.3±2.1	6.5±1.5	0.79	0.667	0.593	0.043
Genus (%)								
Clostridium sensu stricto 1	13.0±2.3	14.1±2.2	14.0±2.4	12.7±1.8	0.90	0.738	0.900	0.057
Methanobrevibacter	8.1±2.5	6.8±1.8	9.2±2.5	10.1±2.6	0.97	0.003	0.786	0.104
Uncultured Porphyromonadaceae	7.6±2.0	6.6±1.9	6.5±2.8	7.7±2.8	0.99	0.969	0.936	0.110
Succinivibrio	5.4±2.4	6.8±1.6	6.7±2.1	5.8±1.5	0.79	0.795	0.586	0.047
Lactobacillus	6.5±2.0^{a}	4.6±2.4^{ab}	3.9±1.8^{b}	5.7±2.2^{ab}	0.86	0.223	0.895	0.005
Christensenellaceae R-7 group	2.8±1.2	4.7±3.8	5.2±1.5	6.1±3.6	1.14	0.022	0.084	0.513
Streptococcus	4.8±3.8	3.6±3.7	1.4±1.2	0.8±0.8	1.12	< 0.001	0.265	0.699
Diversity index								
Simpson's index	0.967	0.970	0.972	0.971	0.002	0.010	0.525	0.148
Principal component scores								
PC1	-13.57	-4.85	7.13	11.29	3.497	< 0.001	0.013	0.360
PC2	-2.01	3.60	0.37	-1.95	4.306	0.605	0.592	0.200

SEM = standard error of the mean; PC = principal component

Each value represents the mean ± SD of 12 replicates; Values with different lowercase superscripts in row are significantly ($p < 0.05$) different based on Tukey's honest significance test.

PC analysis was used to evaluate the variation in the overall microbial composition among different age and treatment groups (Fig. 1). The results showed distinct clusters along PC1, with significant effects of both time ($p < 0.001$) and treatment ($p = 0.013$) detected (Table 5). The samples from the probiotic group on day 45 clustered closer to those on day 66, indicating that the age-related shift of microbiota might have occurred earlier in this group.

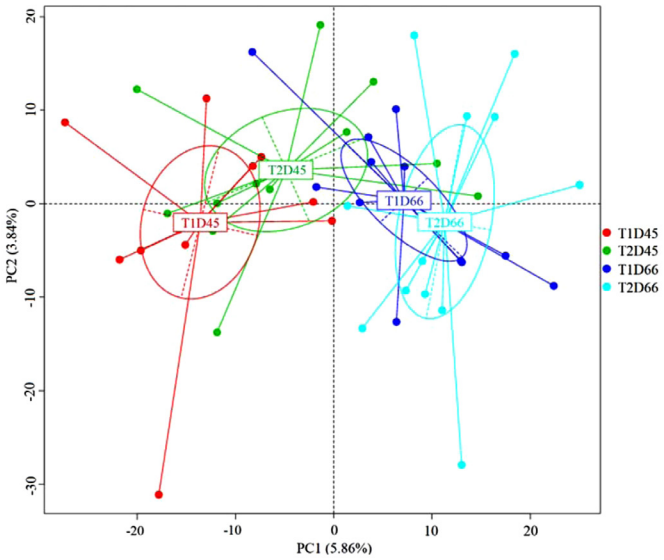

Fig. 1 Principal component (PC) analysis of fecal microbiota of grower and finisher pigs receiving control or probiotic diets, for fecal samples from grower and finisher pigs taken on days 45 and 66 of the experiment, respectively. T1D45 = grower pigs receiving control diet; T2D45 = grower pigs receiving probiotic diet; T1D66 = finisher pigs receiving control diet; T2D66 = finisher pigs receiving probiotic diet; PC = principal component

Discussion

Growth performance, nutrient digestibility, slurry pH and ammonia emission

Supplementation of probiotic improved the growth performance of the pigs during the late-grower and finisher periods. Improvement of nutrient digestibility for days 51–53 coincided with an improvement in the ADG during this period as well. Similar effects of probiotics on the nutrient digestibility and growth performance of grower-finisher pigs have been reported by other studies (Giang et al., 2011; Balasubramanian et al., 2016).

Increased nutrient digestibility and low manure pH have been suggested as the mechanisms of reduced odor emission in farm animals (Arogo et al., 2003; Ferket et al., 2022). Despite the improvements in the nutrient digestibility and decreased slurry pH observed in the current study, ammonia gas emission was not significantly ($p > 0.05$) different between groups. Balasubramanian et al. (2016) also reported improvement in the nutrient digestibility in grower-finisher pigs with the use of a *Bacillus* probiotic; however, no difference was observed in the fecal ammonia gas emission. On the other hand, Wang et al. (2009) reported a reduction in slurry ammonia emission by feeding *Bacillus* a probiotic and concluded that the decline in slurry pH could have been the mechanism of reduced ammonia emission. Reduction of ammonia by feeding *B. subtilis* C-3102 has been reported in studies with other animal species (Blair et al., 2004; Félix et al., 2010; Schauf et al., 2019). One possible reason for not detecting a significant difference in the current study could be the large variation observed in the ammonia gas measurements. Further investigation would be needed before making conclusions on the effect of *B. subtilis* C-3102 on the ammonia gas emission from pig manure.

Age-dependent change of fecal microbiota

An age-dependent shift of fecal microbiota was detected from the grower to the finisher period. Microbial diversity increased with age, with the taxonomic analysis identifying the bacterial groups that were differently represented between the two stages. At the phylum level, relative abundance of the Firmicutes decreased and that of the Euryarchaeota increased with age. At the genus level, *Methanobrevibacter* and the Christensenellaceae R-7 group increased with age. Co-occurrence of methanogens and the Christensenellaceae has been frequently reported in humans, indicating the close relationship between these two bacterial groups in the microbiota (Hansen et al., 2011; Goodrich et al., 2014; Upadhyaya et al., 2016; Waters and Ley, 2019). *Methanobrevibacter* is known to form a hydrogen-based syntrophic relationship with saccharolytic bacteria and enhances energy harvest from the diet (Samuel and Gordon, 2006). The Christensenellaceae is a recently described bacterial family that produces short chain fatty acids (SCFAs) from carbohydrate degradation and is associated with gut health (Waters and Ley, 2019). Both *Methanobrevibacter* and the Christensenellaceae have been reported to be enriched in pigs with higher feed efficiency (McCormack et al., 2017; Quan et al., 2018; Gardiner et al., 2020). In addition, higher bacterial diversity has been reported to be associated with higher feed efficiency in pigs

(Gardiner et al., 2020). Overall, it could be concluded that the development of microbiota from the grower to the finisher period observed in the current study was characterized by a higher capacity for polysaccharide degradation and efficient energy harvest.

The fecal culture analysis showed increases in *E. coli* and *C. perfringens* with age. Both these species are opportunistic pathogens that may cause enteritis, particularly in younger piglets. For example, Chan et al. (2012) studied the prevalence of *C. perfringens* in pigs at different production stages and showed that the number of *C. perfringens* declined with age. However, other researchers enumerated fecal *E. coli* levels in grower and finisher pigs and detected no age-related increase (Giang et al., 2011; Balasubramanian et al., 2016). It is not clear why the age-dependent increase was observed in the current study, which could have been due to disturbance of the gut microbiota from stress. It has been reported that mild handling stress, such as in weight measurement procedures, increased the *E. coli* population in finisher pigs (Dowd et al., 2007). During the current study, the pigs were individually weighed four times and fecal samples were collected twice and this repeated handling stress might have affected the gut microbiota.

Treatment-dependent change of fecal microbiota

The added probiotic influenced the overall composition of the fecal microbiota in the grower-finisher pigs, indicating the possibility that the probiotic-fed grower pigs developed a mature microbiota composition similar to finisher pigs. As the microbiota of the finisher pigs appeared to have higher ability to degrade complex polysaccharides into SCFAs than the grower pigs, the change observed in the probiotic-fed grower pigs could have been due to the improved FCR during this period.

The number of *E. coli* was lower in the probiotic group throughout the study. Although *Lactobacillus* showed a different trend in the grower and finisher periods, notably, the probiotic group had higher *Lactobacillus* numbers in the latter period. *Lactobacillus* is one of the most well-studied gut bacteria and is known to confer numerous benefits in pigs, including improved performance, prevention of diarrhea, stress alleviation and immunomodulation (Hou et al., 2015). An increase of *Lactobacillus* in the gut could have had a positive influence on the probiotic-fed pigs in a later stage where the pigs tend to get more stressed. A decrease in *E. coli* and an increase in *Lactobacillus* levels in pigs fed with probiotics has been reported also by other researchers (Maruta et al., 1996; Giang et al., 2011; Balasubramanian et al., 2016).

In conclusion, inclusion of *B. subtilis* C-3102 in the diet had positive effects on the gut microbiota of grower-finisher pigs. The extensive bacterial analysis performed in the current study that was molecular- and culture-based, provided some novel insights into the complex gut bacterial community of grower-finisher pigs. The alteration of gut microbiota observed in the current study could have contributed to better growth performance and nutrient digestibility, as well as a lower slurry pH for the probiotic-fed pigs.

Conflict of Interest

Dr. Noriko Nakamura, Ms. Suchavadee Sintho, Ms. Napat Pongkijvorasin and Dr. Bhimabol Khongto—are employees of Asahi Biocycle Co., Ltd., the company that owns the product Calsporin®. Ms. Takawan Sooksridang is an employee of Bangkok Animal Research Center Co., Ltd.

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