Effects of *Bacillus subtilis* KN-42 on Growth Performance, Diarrhea and Faecal Bacterial Flora of Weaned Piglets

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ABSTRACT: This research focused on the effects of different doses of *Bacillus subtilis* KN-42 on the growth performance, diarrhea incidence, faecal bacterial flora, and the relative number of *Lactobacillus* and *Escherichia coli* in faeces of weaned piglets to determine whether the strain can serve as a candidate antimicrobial growth promoter. A total of 360 piglets (initial body weight 7.14±0.63 kg) weaned at 26±2 days of age were randomly allotted to 5 treatment groups (4 pens per treatment with 18 pigs per pen) for a 28-day trial. Dietary treatments were basal diet without any antimicrobial (negative control; NC), basal diet supplemented with 120 mg/kg feed of neomycin sulfate (positive control; PC) and basal diet supplemented with 2×10^9 (L), 4×10^9 (M) and 20×10^9 (H) CFU/kg feed of *B. subtilis* KN-42. During the overall period, average daily gain and feed efficiency of piglets were higher in groups PC, M, and H than those in group NC (p<0.05), and all probiotics and antibiotics groups had a lower diarrhea index than group NC (p<0.05). The 16S rDNA gene-based methods were used to analyze faecal bacterial flora on day 28 of experiment. The result of denaturing gradient gel electrophoresis analysis showed that supplementation of *B. subtilis* KN-42 to the diet changed the bacterial communities, with a higher bacterial diversity and band number in group M than in the other four groups. Real-time polymerase chain reaction analysis showed that the relative number of *Lactobacillus* were higher in groups PC and H than in group NC (p<0.05), and the supplemented *B. subtilis* KN-42 to the diet also reduced the relative number of *E. coli* (p<0.05). These results suggest that dietary addition of *B. subtilis* KN-42 can improve the growth performance and gastrointestinal health of piglets. (**Key Words:** *Bacillus subtilis*, Bacterial Community, Diarrhea, Growth Performance, *Lactobacillus*, Weaned Piglets)

INTRODUCTION

The health state of weaned piglets has an enormous impact on their subsequent performance in adaptation to nutritional, psychological and environmental stressors. These stressors may result in low feed intake, low weight gain and poor health, and thus growth-promoting antibiotics are usually used to improve animal growth and health. However, recent studies have found the horizontal transfer of antibiotic resistance genes between bacteria from farm animals, human food and humans (Smillie et al., 2011; Hu et al., 2013). Concerns about transference of antibiotic resistance genes from animals to humans led to withdraw approval for antibiotics as growth promoters in the European Union in 2006. Currently, more countries are making a greater effort to ban on the use of antibiotics as animal growth promoters.

Probiotics are defined as live microorganisms that produce a health benefit when administered in adequate amounts to animals, including humans (Sindhu and Khetarpaul, 2003). They are potential alternatives to growth promoting antibiotics which can affect the health of pigs. Among several bacterial species used as probiotics, *Bacillus subtilis* was once thought to be a strict aerobe, but now known as a facultative anaerobe, which is preferred due to the high resistance of its spores to harsh environment and long-term storage at ambient temperature (Nakano and Zuber, 1998; Hong et al., 2005). The intestinal microflora has been suggested to play an important role in the growth

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of weaned piglets, and *B. subtilis* is an intestinal microorganism that may grow in the gut and consume the oxygen to maintain an anaerobic environment for the prevention or therapy of gastrointestinal disorders.

Many previous studies have reported that dietary supplementation of *B. subtilis* could have some beneficial effects on digestibility and intestinal microbes, thus improving the growth performance of animals (Aliakbarpour et al., 2012; Kim et al., 2012; Sen et al., 2012; Zhang et al., 2012; Tsukahara et al., 2013). Furthermore, *B. subtilis* LS 1-2 is reported to have wide-ranging effects on the intestinal morphology, microbial population and immune status of weanling pigs (Lee et al., 2014). However, it was also found that animals supplemented with probiotics did not always result in better growth performance (Lee et al., 2010). The effect of probiotics depends on the combination of selected bacterial genera, their doses, and feed composition (Vondruskova et al., 2010).

The goal of this study was to investigate the effect of *B. subtilis* KN-42 on the growth performance, diarrhea incidence, faecal *Lactobacillus*, *Escherichia coli* and bacterial diversity of weaned piglets. Denaturing gradient gel electrophoresis (DGGE) was used to characterize the bacterial diversity of faeces, and real-time polymerase chain reaction (PCR) was used to measure the copy number of *Lactobacillus* and *E. coli*.

MATERIALS AND METHODS

Probiotics

The *B. subtilis* KN-42 (CCTCC No: M 208249) was certified as feed additives by the Ministry of Agriculture of People's Republic of China (No: [2009] 2563). The product was composed of spray-dried spore-forming *B. subtilis* containing at least 20×10^9 CFU/g and was donated by a commercial company (Kenuo Biotechnology CO., LTD., Wuhan, China).

Animals and experimental design

Piglets (Duroc×[Landrace×Yorkshire], 82 litters) were obtained from a farm with 3,000 sows (Hainan Agri-Farming Animal Husbandry Group, Haikou, China). At weaning, a total of 360 healthy piglets (initial body weight 7.14±0.63 kg; 26±2 days of age) were selected for a 28-day trial. The piglets were randomly divided into 20 pens, balanced for sex, body weight and litter origin, with 18 piglets in each pen (male: female, 1:1). According to a completely randomized design, the piglets were allotted to 5 treatments with 4 replicates. Dietary treatments were basal diet without any antimicrobial (negative control; NC), basal diet supplemented with 120 mg/kg feed of neomycin sulfate (positive control; PC) and basal diet supplemented with

2×10^9 (L), 4×10^9 (M) and 20×10^9 (H) CFU/kg feed of *B. subtilis* KN-42.

Experimental diets were fed in two phases (phase I: d 1 to 14 and phase II: d 15 to 28 post weaning; Table 1), and all diets met or exceeded nutrients requirement recommended by NRC (1998). During the experiment, all piglets were housed in a temperature-controlled nursery room (25±2°C). Feed and water were available *ad libitum*. All piglets were vaccinated against pseudorabies, foot-and-mouth disease, circovirus, blue-ear disease, asthma and hog cholera before the experiment.

Feed intake and body weight were measured at the end of each phase to determine average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G/F). During the experiment, all piglets were checked daily, and those that had loose faeces (pasty, thick, fluid or watery) were recorded as diarrheal pigs (Giang et al., 2012). The incidence of diarrhea (%) was calculated by the total

Table 1. Ingredient and chemical composition of basal diets

Item	Phase I (d 1 to 14)	Phase II (d 15 to 28)
Ingredient (%)		
Corn, yellow	47.59	60.13
Soybean meal (43% CP)	10.00	16.00
Spray dried plasma protein	3.50	-
Fish meal	5.00	5.00
Dried whey	26.50	14.00
Dicalcium phosphate	1.05	1.40
Limestone	0.15	0.15
L-lysine-HCL (98%)	0.39	0.43
DL-methionine (99%)	0.17	0.14
Sugar	3.00	-
Salt	0.20	0.30
Corn starch	1.40	1.40
Vitamin/mineral premix[1]	1.05	1.05
Chemical composition		
ME (kcal/kg)	3645	3458
CP (%)	20.5	19.0
Lys (%)	1.60	1.48
Met (%)	0.91	0.80
Ca (%)	0.69	0.59
P (%)	0.50	0.45

CP, crude protein; ME, metabolizable energy.

Dietary treatments: NC (negative control, basal diet without any antimicrobial); PC (positive control, diet supplemented with 120 mg/kg of neomycin sulfate); L, M, H (diets supplemented with probiotics 2×10^9, 4×10^9 and 20×10^9 CFU/kg feed, respectively); Antibiotics and probiotic products were added to the diets at the expense of corn starch.

[1] Provided the following per kg of diet: 12,800 IU vitamin A, 4,000 IU vitamin D_3, 80 mg vitamin E, 4 mg vitamin B_1, 10 mg vitamin B_2, 6 mg vitamin B_6, 46 μg vitamin B_{12}, 4 mg vitamin K_3, 20 mg pantothenic acid, 40 mg nicotinic acid, 0.36 mg biotin, 2 mg folic acid, 500 mg choline chloride; 80 mg Mn ($MnSO_4$), 200 mg Fe ($FeSO_4$), 40 mg Cu ($CuSO_4$), 120 mg Zn ($ZnSO_4$), 0.4 mg I ($Ca(IO_3)_2$), 0.25 mg Co ($CoSO_4$), and 0.4 mg Se (Na_2SeO_3).

number of diarrheal piglets over a period divided by the number of piglets and days in that period multiplied by 100. On day 28 of the experiment, faecal samples (5 piglets for each treatment, at least 1 piglet per pen) were collected by rectal massage and stored at −80℃.

DNA extraction and polymerase chain reaction-denaturing gradient gel electrophoresis

The DNA from faecal samples was extracted with the QIAamp DNA Stool Mini Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions, at 95℃ for the initial lysis step (Li et al., 2003). DNA was eluted from the matrix using 100 μL elution buffer, and stored at −20℃ before use. DNA concentration and quantity were tested on a Nanodrop ND-100 spectrophotometer (Thermo, Wilmington, DE, USA).

The 50 μL PCR reaction mixture contained 5 μL of 10×PCR buffer (Takara, Dalian, China), 2 μL of dNTPs (2.5 mM of each, Takara), 1 μL of each primer (10 μM), 2.5 U of Taq polymerase (Takara) and 50 ng template DNA. The primers were 968F-GC and 1401R (Table 2; Invitrogen, Shanghai, China) targeted to the V6~V8 regions of 16S rDNA. The DNA was amplified under following conditions: 1 cycle for 7 min at 94℃, 35 cycles of (94℃ for 30 s, 56℃ for 20 s, 68℃ for 30 s) and a final elongation of 7 min at 68℃ in the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR products from the same group (5 piglets) were mixed together as one sample. Before running on DGGE gels, the PCR products were cleaned using a PCR Purification Kit (Omega, Norcross, GA, USA).

The DGGE analysis was performed using the DCode Universal Mutation Detection System (Bio-Rad) as described by Walter et al. (2000) with the following modifications. Using a denaturant gradient range from 42% to 58%, 10 μL of purified DNA samples (32 ng/ μL) was subjected to electrophoresis at 10 V for 10 min at 60℃ and subsequently at 85 V for 16 h in 0.5×TAE buffer at 60℃. The DNA bands in gels were visualized by silver staining (van Orsouw et al., 1997).

Denaturing gradient gel electrophoresis band sequencing and analysis

Excision and purification of DNA fragments from DGGE gels were performed as described by Ben Omar and Ampe (2000). The purified DNA (5 μL) was amplified using the same primers and reaction conditions as previously described except that the GC clamp was excluded from the forward primer (Table 2). The PCR products were cleaned using a PCR Purification Kit (Omega) and sequenced by Invitrogen (Shanghai, China) using an ABI Prism 377 sequencer (ABI, Foster, CA, USA). The closest relatives were identified by comparing newly determined sequences with those available in the V6–V8 regions of the 16S rDNA gene sequences in the GenBank DNA database (www.ncbi.nih.gov). The identities of the relatives were determined on the basis of the highest score.

Quantity One (version 4.6.2; Bio-Rad) was used for band analysis. The number of bands detected in a DGGE lane was used as a measure of the number of species present (species richness, S). For each sample, diversity indices were calculated based on the gauss trace quality, which was determined using background subtraction as an estimate of the relative population size of each species. Shannon-wiener diversity index (H′), species evenness (J), Berger-Parker index (d) and Simpson's index (1/D) were calculated using the software BIO-DAP (Fundy National Park, Canada).

Quantitative real-time polymerase chain reaction analysis

Quantitative real-time PCR (qPCR) was performed using previously described primer sets and annealing temperatures (Table 2). Counts for total bacteria, *Lactobacillus* and *E. coli* were obtained. Amplification was performed in an Applied Biosystems ViiA 7 Real-time PCR System (ABI) using an iTaq Universal SYBR Green Supermix (Bio-Rad) as follows: 1 cycle at 95℃ for 10 min, and 40 cycles of (95℃ for 15 s, 50℃ to 60℃ [Table 2] for 20 s and 72℃ for 30 s). The data were collected at the extension step (72℃ for 30 s). Standard curves were generated using serial dilutions (10^2 to 10^7) of purified

Table 2. Primers used for denaturing gradient gel electrophoresis (DGGE) and real-time PCR

Target group	Prime sequence (5′→3′)	Amplicon size (bp)	Annealing temp (℃)	Reference
Total bacteria of DGGE	AACGCGAAGAACCTTAC (968F-GC[1]) CGGTGTGTACAAGACCC (1401R)	435	56	Ricca et al. (2010)
Total bacteria	CGGYCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	200	60	Lee et al. (1996)
Lactobacillus	CGATGAGTGCTAGGTGTTGGA CAAGATGTCAAGACCTGGTAAG	186	60	Fu et al. (2006)
Escherichia coli	CAATGGTGATGTCAGCGTT ACACTCTGTCCGGCTTTTG	163	58	Srinivasan et al. (2011)

PCR, polymerase chain reaction
[1] GC clamp (5′-CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGG-3′).

Table 3. Average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (G/F) of weaned piglets[1] fed diet supplemented with antibiotics or *B. subtilis*

Item	NC	PC	L[2]	M[2]	H[2]	SEM	p value
Initial weight (kg)	7.22	7.08	7.18	7.07	7.14	0.15	0.998
Phase I (d 1 to 14)							
ADG (g/d)	245	249	257	259	275	3.73	0.074
ADFI (g/d)	378	356	362	368	365	3.50	0.425
G/F (g/kg)	648^c	696^{bc}	710^{ab}	704^{ab}	754^a	9.17	<0.001
Phase II (d 15 to 28)							
ADG (g/d)	353^b	411^a	384^{ab}	404^a	389^a	5.51	0.001
ADFI (g/d)	676	720	728	721	717	8.66	0.359
G/F (g/kg)	522^c	571^a	528^{bc}	560^{ab}	543^{abc}	5.36	0.004
Overall (d 1 to 28)							
ADG (g/d)	299^b	330^a	321^{ab}	331^a	332^a	3.72	0.006
ADFI (g/d)	527	539	545	545	541	5.14	0.837
G/F (g/kg)	567^b	612^a	588^{ab}	609^a	614^a	4.97	0.001

NC, negative control, basal diet; PC, positive control, diet supplemented with antibiotics; SEM, standard error of the mean.

[1] 20 pens of piglets (18 piglets per pen) with pen as the experimental unit, and each mean based on 4 replicates.

[2] L, M, H = diets supplemented with probiotics 2×10^9, 4×10^9, and 20×10^9 CFU/kg feed, respectively.

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

genomic DNA obtained by standard PCR with corresponding primers. After amplification, the melting curves were checked to confirm the amplification results. The total counts were expressed in $\log_{10}$ gene copy number/μL, whereas the values for the other bacterial groups were expressed in relative numbers versus total bacteria.

Statistical analyses

All data of the experiment were analyzed using the general linear model procedure of SAS (version 8.0; SAS Inst. Inc., Cary, NC, USA), with pen as the experimental unit. The bacterial counts were transformed ($\log_{10}$) before statistical analysis. Statistical differences among treatments were separated by Tukey's HSD test, and considered significant at p values <0.05.

RESULTS

Growth performance

During phase I, group H significantly improved the G/F as compared to groups NC and PC (p<0.05), and groups L and M had a higher G/F than group NC (p<0.05; Table 3),

but no significant difference was found in ADG among 5 groups (p>0.05). During phase II, groups PC and M improved ADG compared with group NC (p<0.05), and there was no significant difference in G/F among groups PC, M, and H. Throughout the experiment, groups PC, M, and H showed improvement in ADG and G/F as compared to group NC (p<0.05). However, no significant difference was found in ADFI among the 5 treatments during phases I, II and overall periods (p>0.05).

Diarrhea incidence

Dietary treatments had a significant impact on the incidence of diarrhea in piglets (Table 4). During phase I, the probiotics groups had a lower incidence of diarrhea than group NC (p<0.05), and no significant difference was observed in diarrhea incidence between group PC and the other four groups. During phase II and throughout the experiment, the piglets fed diets supplemented with antibiotics or probiotics significantly decreased the incidence of diarrhea compared with the negative control (p<0.05), and there was no significant difference in the incidence of diarrhea among groups PC, M, and H.

Table 4. Diarrhea incidence[1] of weaned piglets fed diet supplemented with antibiotics or *B. subtilis*

Item	NC	PC	L[2]	M[2]	H[2]	SEM	p value
Phase I (d 1 to 14,%)	4.25^a	3.37^{ab}	2.27^b	2.78^b	2.22^b	0.24	0.016
Phase II (d 15 to 28, %)	8.80^a	2.51^c	5.21^b	3.71^{bc}	3.06^{bc}	0.57	<0.001
Overall (d 1 to 28, %)	6.53^a	2.94^b	3.74^b	3.24^b	2.64^b	0.36	<0.001

NC, negative control, basal diet; PC, positive control, diet supplemented with antibiotics; SEM, standard error of the mean.

[1] Diarrhea incidence (%) = the total number of diarrheal piglets over a period divided by the number of piglets and days in that period multiplied by 100.

[2] L, M, H = diets supplemented with probiotics 2×10^9, 4×10^9, and 20×10^9 CFU/kg feed, respectively.

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

Bacterial diversity based on polymerase chain reaction-denaturing gradient gel electrophoresis

Figure 1 shows the DGGE fingerprinting profile of the bacterial communities obtained from the 5 samples and their similarities generated by the unweighted pair-group method with arithmetic means (UPGMA). As displayed in Figure 1a, some differences were observed in predominant bands among the 5 groups. However, Figure 1b showed a 75% similarity in the bacterial community structures between groups NC and M as well as groups PC and H, demonstrating that the supplementation of probiotics to the diet changed the bacterial community.

As shown in Table 5, in terms of overall species richness (as estimated by the number of major bands present in DGGE profiles), the piglets supplemented with a medium and a high dose of probiotics (groups M and H) exhibited a higher number of bands than those of groups NC, PC, and L. As for the Shannon-wiener diversity index H′ for the bacterial community in the five groups, group M (2.93) appeared to have a higher bacterial diversity than the others, while group L showed the lowest bacterial diversity (2.52), which was close to group PC (2.59). For the Simpson's index (1/D), the results of bacterial diversity were similar to H′. Additionally, no significant difference was found in the species evenness index, but group L was found to have the highest Berger-Parker index in the five groups. The results showed that a right dose of probiotics improved the bacterial community, but a high or low dose reduced the bacterial diversity.

Predominant bands (total number 25, marked with

Table 5. Bacterial diversity index calculated from the DGGE banding patterns (Figure 1a)

Index	NC	PC	L[1]	M[1]	H[1]
Species richness (S)	23	19	18	27	24
Shannon's index (H′)	2.8	2.59	2.52	2.93	2.82
Species evenness (J)	0.89	0.88	0.87	0.89	0.89
Berger-Parker index (d)	0.152	0.145	0.173	0.119	0.131
Simpson's index (1/D)[2]	13.18	11.21	9.98	15.47	13.40

NC, negative control, basal diet; PC, positive control, diet supplemented with antibiotics); SEM, standard error of the mean.

[1] L, M, H = diets supplemented with probiotics 2×10^9, 4×10^9 and 20×10^9 CFU/kg feed, respectively.

[2] 1/D, reciprocal of Simpson's diversity index.

numbers in Figure 1a) were excised and re-amplified to identify species in the samples (Table 6). The sequence similarity of each band was ≥97% (except 94% for band 23) as compared with that available in GenBank database. As shown in Figure 1a, major differences are present in bands 3, 7, 12, 15, 22, 23, 24, 25, and 30. When compared to the negative control NC, the antibiotics promoted the growth of bacteria related to uncultured bacteria (15), *Lactobacillus amylovorus* (23), uncultured *Lachnospiraceae* bacterium (24) and *Lactobacillus kitasatoni* (30), and inhibited the bacteria related to *Eubacterium eligens* (3), uncultured bacterium (7) and *Ruminococcus* sp. (22). The probiotics groups M and H promoted the bacteria related to *Eubacterium coprostanoligenes* (12), *L. amylovorus* (23), uncultured *Lachnospiraceae* bacterium (24) and *L. kitasatoni* (30). In lane H, a new band 25 related to *Faecalibacterium prausnitzii* was found as compared to the
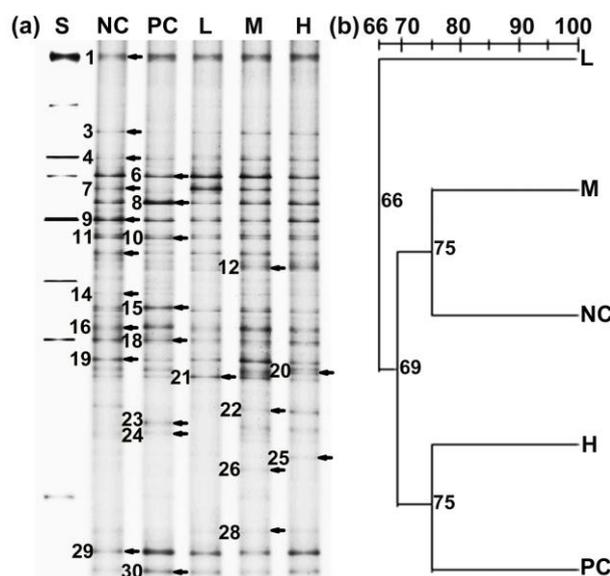

Figure 1. Bacterial community of weaned piglets fed diet supplemented with antibiotics or *B. subtilis*. (a) DGGE profiles of the V6 to V8 regions of the 16S rDNA gene fragments from the samples. The denaturant gradient range is 42% to 58% and the major difference bands are numbered. Lane S (Standard ladder, which indicates PCR products generated from different bacterial 16S rDNA genes with primers 968F-GC and 1401R); NC (negative control, basal diet); PC (positive control, diet supplemented with antibiotics); L, M, H (diets supplemented with probiotics 2×10^9, 4×10^9, and 20×10^9 CFU/kg feed, respectively); (b) Unweighted pair-group method with arithmetic means (UPGMA) analysis of Dice similarity indices from DGGE profiles. DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction.

Table 6. Identification of band fragments in DGGE gels (Figure 1a)

Band no.[1]	Closest relative and NCBI accession number	Identity (%)[2]
1	Uncultured bacterium (JQ187036.1)	99
3	*Eubacterium eligens* ATCC 27750 (CP001104.1)	97
4	Uncultured bacterium (NR_025207.1)	97
6	*Robinsoniella peoriensis* (AF445283.2)	96
7	Uncultured bacterium (HQ716245.1)	100
8	*Ruminococcus* sp. (FJ611794.2)	100
9	*Clostridium irregulare* (JX898025.1)	97
10	Uncultured bacterium (JQ820130.1)	100
11	*Clostridiaceae* bacterium (EU728782.1)	98
12	*Eubacterium coprostanoligenes* (HM037995.1)	99
14	Swine manure bacterium (AY167964.1)	99
15	Uncultured bacterium (FP077070.1)	100
16	Uncultured *Clostridium* sp. (GQ868439.1)	99
18	Uncultured *Firmicutes* bacterium (JN568109.1)	100
19	*Lachnospiraceae* bacterium (EU728751.1)	99
20	*Ruminococcus obeum* (AY169411.1)	100
21	Uncultured bacterium (EU472437.1)	97
22	*Ruminococcus* sp. (EU728789.1)	97
23	*Lactobacillus amylovorus* (CP002609.1)	94
24	Uncultured *Lachnospiraceae* bacterium (JX230492.1)	100
25	*Faecalibacterium prausnitzii* (AY169430.1)	99
26	Uncultured bacterium clone (FJ880520.1)	97
28	*Ruminococcus* sp. (EU728790.1)	99
29	*Lactobacillus reuteri* (CP006603.1)	99
30	*Lactobacillus kitasatonis* DSM 16761T (FR683090.1)	98

DGGE, denaturing gradient gel electrophoresis.

[1] Bands are numbered according to Figure 1a. [2] Identity represents the sequence identity (%) compared with that in the GenBank database.

Quantitative real-time polymerase chain reaction analysis

The quantification of 16S rDNA gene copy numbers from faecal samples revealed that densities of *Lactobacillus* were significantly higher in groups H and PC than in the negative control (p<0.05), and the relative number of *Lactobacillus* increased with an increasing dose of probiotics, indicating that the antibiotics and probiotics improved the growth of *Lactobacillus* (Table 7). In addition, the supplementation of antibiotics and probiotics significantly decreased the relative number of *E. coli* (p<0.05), but no significant difference was observed in the relative densities of total bacteria (p>0.05).

DISCUSSION

Several strains of *B. subtilis* can be used as feed additives, and no safety concerns are identified when used in direct-fed microbial products (Sen et al., 2012; Cui et al., 2013; Lee et al., 2014). We hypothesized that *B. subtilis* would help the balance of microflora by stimulating the beneficial bacteria, thereby improving gut health, reducing diarrhea incidence indirectly, and enhancing the growth performance. The PCR-DGGE has been successfully used to study the taxonomy of bacterial communities in the pigs (Petersson et al., 2009; Han et al., 2011). The microbiota of

Table 7. Real-time PCR analysis of total bacterial counts and relative contributions of 5 bacterial groups[1]

Item	NC	PC	L[2]	M[2]	H[3]	SEM	p value
Total bacteria (log[10] copies/µL)	8.11	8.33	8.27	8.17	8.21	0.04	0.372
Lactobacillus (%)	15.40[b]	28.85[a]	15.75[b]	17.70[ab]	29.60[a]	1.81	0.004
Escherichia coli (10^{-2}, %)	5.25[a]	0.87[b]	2.26[b]	1.71[b]	2.27[b]	0.36	<0.001

PCR, polymerase chain reaction; NC, negative control, basal diet; PC, positive control, diet supplemented with antibiotics; SEM, standard error of the mean.

[1] Faecal samples were taken from 5 weaned piglets per treatment.

[2] L, M, H = diets supplemented with probiotics 2×10^9, 4×10^9, and 20×10^9 CFU/kg feed, respectively.

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

animals usually show considerable variations between individuals (Petersson et al., 2009), and whether these variations are due to different treatments or to naturally occurring deviations is not always known (Gong et al., 2008). In order to reduce individual differences in microbiota, we mixed the 5 samples from the same group together for DGGE analysis. Additionally, qPCR was used to investigate the precise and profound changes in the abundances of *Lactobacillus* and *E. coli*.

Weaning is a stressful period in the life cycle of pigs, which is associated with changes in diet, gut environment and gut morphology, and thus may result in low growth rate, high diarrhea incidence and imbalanced intestinal microecology (Giang et al., 2012). These problems can be reduced by supplementing the diet with probiotics. Previous studies reported that the addition of *B. subtilis* var. *natto* improved the growth and feed intake of geese (Chen et al., 2013), and the diets supplemented with 10^8 CFU/kg feed of *B. subtilis* improved the ADG of broilers (Zhang et al., 2012). Other researchers also reported that the supplementation of *B. subtilis* to diet improved the growth performance of pigs (Alexopoulos et al., 2004; Wang et al., 2011; Lee et al., 2014). However, several studies failed to find its positive effects on the growth performance of animals (Willis and Reid, 2008; Lee et al., 2010).

The growth performance may be linked to diarrhea incidence, and thus the diarrhea incidence of piglets was recorded daily in this study. The data indicated that diets supplemented with probiotics significantly reduced the incidence of diarrhea of piglets, which is consistent with several previous studies. It is reported that supplementation of probiotics to pig diets decreased the diarrhea score (Alexopoulos et al., 2004), and induced a 59% decrease in diarrhea incidence (Taras et al., 2005). Also, a previous study suggested that *B. subtilis* reduced *Citrobacter rodentium*-induced diarrhea of mice (Jones and Knight, 2012). It can be deduced from these results that *B. subtilis* can effectively prevent diarrhea in weaned piglets.

Lactobacillus is considered as a beneficial bacterium for the balance of intestinal microbiota, due to its health-promoting effects such as prevention of diarrhea and intestinal infections. A previous study on intestinal microbiota of weaned piglets has shown that after weaning, *E. coli* concentrations increased while the number of *Lactobacillus* decreased (Konstantinov et al., 2006). In this study, the result of qPCR analysis showed that the relative number of *Lactobacillus* increased in piglets treated with antibiotics and probiotics, and DGGE profiles also showed the number of bacteria related to *L. amylovorus* and *L. kitasatoni* increased. Neomycin sulfate usually inhibits the growth of gram-negative bacteria and some of the gram-positive bacteria, such as *Escherichia*, *Klebsiella* and

Corynebacterium. However, the genus *Lactobacillus* belongs to the gram-positive bacteria which are not the main bacteria inhibited by Neomycin sulfate. It was reported that pigs supplemented with antibiotics had a higher *Lactobacillus* in the ileum than the control (Li et al., 2008). Previous studies have also found that the compound probiotics containing *B. subtilis* increased lactic acid bacteria (Giang et al., 2012), and the number of *Lactobacillus* was significantly greater in the probiotics groups than in the control (Han et al., 2013). Additionally, it has been reported that an increment of *Lactobacillus* results in a relative decrease in other bacteria such as *Clostridia* and Coliforms (Vanhoutte et al., 2006).

As we know, *E. coli* is one of the major sources of intestinal pathogens, and a few strains can induce serious illness, including diarrhea. In the post-weaning period, supplementation of probiotics to pig diets is essential for the prophylaxis of diarrhea, which is usually induced by enterotoxigenic *E. coli* strains. A previous study reported that oral administration of *B. subtilis* DB9011 in weaned piglets inhibited the growth of Shiga toxin 2e-producing *E. coli* in the ileum (Tsukahara et al., 2013). The probiotics were also found to reduce *E. coli* counts in the intestine of piglets (Giang et al., 2012). Therefore, an increase of *Lactobacillus* and a decrease of *E. coli* may, to a degree, result in a lower diarrhea incidence in antibiotics and probiotics groups.

The bacterial diversity could represent a benefit for the weaned animals because of the possible link between the diversity of ecosystems and their ability to respond to perturbations (McCann, 2000). The diversity of the microbiota is usually represented by the number of identifiable DGGE bands. The results showed that group M had the highest number of bands and the highest bacterial diversity in the five groups. Taras et al. (2007) reported that the bacterial community of pigs could be modified by supplementation of *E. faecium*, and Pieper et al. (2009) found that piglets treated with *Lactobacillus plantarum* increased the Simpson's diversity index. Furthermore, *B. subtilis* was observed to have beneficial effects on caecal microflora in broiler chicks (Sen et al., 2011).

Most of the species identified from the DGGE profiles are uncultured bacteria, and largely belong to *Firmicutes*. It is found that most of the intestinal microbes cannot be cultured, and *Firmicutes* constitute a large portion of the faecal community (Eckburg et al., 2005). In this study, the major difference bands related to culturable bacteria are *E. eligens*, *E. coprostanoligenes*, *Ruminococcus* sp., *L. amylovorus*, *L. kitasatonis* and *F. prausnitzii*. Among them, *E. eligens* is known to belong to *Clostridium* Cluster XIVa, which is one of the most common gut *Firmicute* clades, and consists of many species producing butyrate, acetate and

lactate (Pryde et al., 2002). *Eubacterium coprostanoligenes*, a small gram-positive and cholesterol-reducing anaerobe, promotes the conversion of cholesterol to coprostanol (Madden et al., 1999). Several strains of *Ruminococcus* were identified to be present as active bacteria and important members of the bacterial community in the human gastrointestinal tract, and beneficial for the digestion of crude fibre (Zoetendal et al., 1998). Interestingly, a band related to *F. prausnitzii*, which was detected in group H. The *F. prausnitzii*, is one of the most abundant commensal bacteria in the healthy human large intestine (Lopez-Siles et al., 2012), and serves as an anti-inflammatory bacterium that can be used for Crohn's disease treatment (Sokol et al., 2008). Whether this major difference bacterium affects the health of piglets is a very complicated issue and remains to be elucidated.

In conclusion, this study has found that *B. subtilis* KN-42 effectively improved the growth performance of piglets and reduced the incidence of diarrhea in the weaned piglets. Furthermore, *B. subtilis* KN-42 also improved the bacterial diversity of the intestinal environment, increased the relative number of *Lactobacillus* and reduced the relative number of *E. coli* in the faeces of weaned piglets. These results suggest that *B. subtilis* KN-42 can serve as an alternative to antibiotics in diets for weaned piglets.

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