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# Screening of *Bacillus* strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of *Bacillus subtilis* MA139 in pigs

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**Abstract** A total of 124 samples were collected from the intestine of broiler chickens, piglet faeces, fermented foods, soils and Chinese herbs. More than 750 strains of aerobic, spore-forming bacteria were isolated from these samples. The inhibitory activity of these spore-forming strains against *Escherichia coli* K88, *E. coli* K99, *Salmonella typhimurium* and *Staphylococcus aureus* was assessed using a disc plate diffusion assay. The six bacilli with the largest inhibition zones against the four indicator bacteria were chosen and assessed for their resistance to unfavorable conditions within simulated gut environments. The strain *Bacillus subtilis* MA139 showed full resistance to pH 2, 0.3% bile salts and exhibited the highest antimicrobial activity. Based on these results, *B. subtilis* MA139 was selected as a potential probiotic and fed to piglets at concentrations of $2.2 \times 10^5$, $2.2 \times 10^6$ or $2.2 \times 10^7$ CFU/g of feed during a 28-day feeding trial. A negative control consisting of the basal diet with no additives and a positive control consisting of the basal diet supplemented with 16 g/ton flavomycin were also included. Ninety piglets between 35 and 40 days old were used in the in vivo animal trials. *B. subtilis* MA139 enhanced daily gain ($P = 0.10$) and feed conversion ($P = 0.03$) compared with the negative control. The performance of pigs fed *B. subtilis* MA139 supplemented diets did not differ from that of pigs fed the antibiotic diet. There was a significant increase in Lactobacilli cell counts ($P = 0.02$) and a numerical decrease in *E. coli* counts ($P = 0.05$) in the faecal samples of pigs fed *B. subtilis* MA139 with $2.2 \times 10^5$ CFU/g of feed. The overall results of this study show that the use of initial co-culture with indicator pathogens, a disc plate diffusion assay and simulated gut environment tolerance tests is one of effective methods of screening *Bacillus* for probiotic use and that *B. subtilis* MA139 is a promising alternative to antibiotics for use as a feed additive in piglet diets.

**Keywords** *Bacillus* · Screening · Antimicrobial activity · Stability · Piglets · Performance

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## Introduction

Due to concerns about residues in animal products and the development of bacterial resistance to antibiotics, the potential exists for the implementation of a complete ban of the use of antibiotics in animal feed. As a consequence, the development of alternatives to antibiotics is receiving considerable attention (Turner et al. 2001). Probiotics are one potential

alternative. Probiotics have been defined as ''live microbial feed supplements that can benefit the host by improving its intestinal balance'' (Fuller 1989). As living microorganisms, they produce no drug resistance or drug residues (Kyriakis et al. 1999; Scharek et al. 2005).

The most common microorganisms found in the probiotic products currently available are lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species, which are resident microflora in the gastrointestinal tract of most animals (Chang et al. 2001; Simpson et al. 2004). *Bacillus* species, a type of exogenous spore-forming bacteria, are not normally found in the gastrointestinal tract but have also been shown to be effective in keeping a favorable balance of microflora in the gastrointestinal tract and in improving animal performance (Zani et al. 1998; Adami and Cavazzoni 1999; Kyriakis et al. 1999; Alexopoulos et al. 2004; Kritas and Morrison 2005). Generally, when a *Bacillus* is used as a probiotic, it is used in the spore form and thus can be resistant to unfavorable conditions arising during transit through the gastrointestinal tract of animals. However, not all strains of *Bacillus* are equally resistant to the environment in the gastrointestinal tract and antimicrobial activity varies between strains (Chang et al. 2001; Dunne et al. 2001; O'Sullivan 2001). Therefore, the selection of *Bacillus* strains for use as potential probiotics could play a crucial role in increasing their effectiveness as feed additives (Barbosa et al. 2005).

The objective of this study was to screen strains of spore-forming bacteria used as probiotics and then evaluate the strain of *Bacillus* showing the greatest in vitro potential for its effectiveness in improving piglet performance and faecal flora.

## Materials and methods

### Bacterial strains, growth media and initial isolation of spore-formers

Four pathogens were used as indicator bacteria, including *Escherichia coli* IVDC C83901, serotype O8:K87, K88ac; *E. coli* IVDC C83529, serotype O141:K99; *Salmonella typhimurium* IVDC C77-31 and *Staphylococcus aureus* IVDC C56005. The indicator bacteria were purchased from the China Veterinary Culture Collection Center (Beijing, China). The bacteria were cultivated and incubated in Nutrient Broth. All strains were stored in −80°C with 20% sterile glycerol until needed.

One hundred and twenty-four samples were collected from the intestine of broiler chickens, piglet faeces, fermented foods, soils and Chinese herbs. About 2 g of each sample was individually cultured in 5 ml of Mixed Nutrient broth (peptone, 5.0 g/l; beef extract, 3.0 g/l; glucose, 5.0 g/l; yeast extract, 1.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l, pH 6.8–7.0) inoculated with all four of the indicator bacteria (about $10^6$ CFU/ml each). The incubation was conducted in 50 ml test tubes placed on a rotary shaker ($n$=250 rpm) at 37°C for 48 h. The *Bacillus* strains subsequently screened for antimicrobial production were isolated as the survivors of a treatment of cultures for 15 min at 80°C by water bath.

The cultures were then cross-streaked in Mixed Nutrient agar (Mixed Nutrient broth plus 1.5% agar) in Petri dishes and grown at 37°C for 24 h. Individual colonies of bacteria that appeared to be distinct and to have grown from one cell were chosen to further detect their antimicrobial activity.

### Antimicrobial activity screening assay

The antimicrobial activity of the initially isolated spore-formers was determined using a disc plate diffusion assay conducted according to the methods of Lyver et al. (1998). Pure isolates of spore-forming bacteria (6 isolates per dish) were spotted with sterile toothpicks on Mixed Nutrient agar (Mixed Nutrient Broth plus 1.5% agar). After culturing at 37°C for 24 h, the isolates were killed by exposure to chloroform vapor for 30 min. The chloroform was then evaporated for 20 min.

0.1 ml of a 24 h culture of *E. coli* K88 in Nutrient Broth (about $10^8$ CFU/ml) was spread on the plate. After 16–18 h incubation at 37°C, the inhibition zones around the spots were measured in both directions and the average inhibition diameter was expressed in millimeters. Based on this, six spore-formers were selected according to their larger inhibition zones against *E. coli* K88. The six strains were further evaluated for their inhibitory activity against all four indicator bacteria by the same method. One strain per Petri dish was spotted on the center of the plate. The concentration of all four indicator bacteria was about $10^8$ CFU/ml. The inhibition zones around

the spots were measured. The antimicrobial activity screening assay was repeated in triplicate for these six strains.

### Preparation of bacterial spores for gastrointestinal tract tolerance assay

The six selected spore-forming bacteria were serially transferred twice and activated in Mixed Nutrient broth. They were cultured aerobically at 37°C for 36 h and then heated at 80°C by water bath for 15 min to kill vegetative cells (Oscariz et al. 1999; Foldes et al. 2000). The surviving spores were collected by centrifugation ($2500 \times g$ for 10 min), washed twice and then suspended in Phosphate-Buffer Saline (PBS; 0.144% $Na_2HPO_4$, 0.024% $KH_2PO_4$, pH 7.0). The total viable count of the washed bacterial spore suspension was determined prior to the gastrointestinal tract tolerance test.

### Simulated gastric fluid tolerance test

A pH of 2 was selected as the critical point to detect the survival of spores in simulated gastric fluid. Simulated gastric fluid was prepared following the method of Huang and Adams (2004). Pepsin was suspended in sterile saline (0.5%, w/v) to a final concentration of 3 g/l, and the pH was adjusted to 2.0 with concentrated HCI or 10% NaOH (w/v) using a pH meter (PHS-3B, Leici Co., China). A 0.5 ml aliquot of each washed spore suspension was added to 4.5 ml simulated gastric fluid (10-fold dilution of the spores) and then vortexed vigorously for 10 s and incubated at 37°C. After 1, 2 and 3 h, 0.5 ml of the spore culture was sampled to determine the number of surviving spores. The spore counts at 0 h were determined by a direct 10-fold dilution in physiological saline.

### Simulated small intestinal fluid tolerance test

Simulated small intestinal fluid was prepared by suspending pancreatin (porcine pancreas, Sigma) in Mixed Nutrient broth to a final concentration of 1 g/l with 0.3% bile salts (w/v) (Oxgall bile B8381, Sigma) (Huang and Adams 2004). The bile tolerance test was conducted following the procedures of Hyronimus et al. (2000). Spores were incubated in 5 ml of simulated intestinal fluid in 50 ml test tubes and cultured on a rotary shaker ($n$ = 250 rpm) for 5 days at 37°C. Simulated small intestinal fluid without inoculation was used as a control.

### Determination of total spore counts

The total counts of viable spores were determined using a 10-fold dilution and plate-counting following incubation on Mixed Nutrient agar plates at 37°C for 24 h.

### Preparation of spores for the animal feeding trial

Based on the in vitro assessment of the six selected isolates, *B. subtilis* MA139 was selected for further analysis and in vivo evaluation in pigs. The strain was identified through standard morphological, biochemical, and physiological tests and by 16S rRNA gene sequence analysis. The GenBank Accession number for the 16S rRNA gene sequence is DQ415893.

*B. subtilis* MA139 was grown aerobically for about 36 h using batch fermentation until the spores were completely formed. Then, the spore forming culture was harvested and mixed with corn meal in a ratio of 1:1 (v/v). The mixture was dried at 40°C for 48 h using ventilation provided by a cabinet drier. The concentration of spores in the preparation was $2.2 \times 10^9$ CFU/g.

### Animal trials

Ninety castrated male, crossbred (Landrace×Large White) piglets, 35–40 days old, were randomly assigned to one of five treatments. The treatments included a negative control consisting of the basal diet with no additives (Group C), a positive control consisting of the basal diet supplemented with 16 g/ton flavomycin (Group A) and three experimental diets consisting of the basal diet supplemented with either 0.1, 1.0 or 10 kg/ton spore preparation. The inclusion of these levels of spores provided $2.2 \times 10^5$, $2.2 \times 10^6$ and $2.2 \times 10^7$ CFU/g feed. The tested dosages were designed based on the results of earlier reports (Zani et al. 1998; Kyriakis et al. 1999; Alexopoulos et al. 2004).

The basal diet was based on corn (60.6%), soybean meal (23.0%), fish meal (6.0%), whey (5.0%) and wheat bran (1.5%). The diet was formulated to provide 3.26 Mcal/kg metabolizable energy (ME),

19.82% crude protein, and 1.23% lysine. Sufficient vitamins and minerals were added to meet the nutrient requirements for the growth of piglets of the weight range studied based on NRC (1998). The spore preparation was added to diets by substituting for corn meal.

The piglets were housed with three piglets per pen in 165 × 170 $cm^2$ raised weaner decks equipped with a mesh floor. Six pens of pigs received each treatment. The pig barn was maintained at 25–28°C. All piglets had free access to feed and water throughout the 4-week feeding trial. Piglets were weighed and the feed intake was recorded every two weeks in order to calculate Average Daily Weight Gain, Average Daily Feed Intake and Feed Conversion Ratio.

Fresh faecal samples were collected from three randomly chosen pens for each treatment on d 0, 14, and 28. The samples were stored in a freezer at −20°C until analysis for the counts of lactobacilli and *E. coli* according to the methods of Mikkelsen et al. (2003). Faeces from the negative control and the diet containing spores that performed best during the performance trial were analyzed.

### Statistical analysis

In the screening experiment, the counts of viable spores were transformed to $\log_{10}$ values. The final results were expressed as the mean and standard deviation of three determinations. The values were compared using student's *t*-test and *P*-values less than 0.05 were regarded as significant. Data in the feeding trial were subjected to a one-way ANOVA using the PROC General Linear Model procedure in SAS (version 8; SAS Institute, Inc., Cary, NC). The pen was considered the experimental unit. Orthogonal comparisons were used to compare the effects of the *Bacillus*-containing diets with both the positive and negative control as well as to compare the two control treatments.

## Results and discussion

### Isolation of spore formers from different sources and their inhibitory activity

Approximate 750 strains of spore-formers were prescreened from the 124 samples. The selected strains shared the typical characteristics of *Bacillus* species, which were gram-positive, rod-shaped, catalase positive and containing spore-forming aerobic bacteria. Pre-screening of *Bacillus* by amplification in broth before agar isolation has been reported previously (Foldes et al. 2000) and we have further developed this approach as in the prescreening trial the collected samples were co-cultured with the indicator strains before isolation in agar plates. This co-culture had two aims: (1) nutrient broth provides a rich environment to allow the candidate bacteria to grow; (2) the indicator strains served as competitors to *Bacillus* and the spore formers with higher antimicrobial activity should survive from the competition environment. A drawback to this method is that if the indicator strains are inoculated too heavily compared to the initial *Bacillus* inoculum then candidate *Bacillus* may be disadvantaged. However, this is compensated by the ability of co-culture to yield *Bacillus* strains with high antimicrobial activities.

The 750 strains of *Bacillus* were tested using the disc plate diffusion assay and there was considerable variability in their ability to inhibit the growth of *E. coli* K88. Based on their high inhibitory activity against *E. coli* K88 during the initial screening, six *Bacillus* strains were selected for further study. These six strains were designated as *Bacillus* spp. 23, 357, 559, 634 and 744 as well as *B. subtilis* MA139. The *Bacillus* spp. 23 and *B. subtilis* MA139 were originally isolated from Chinese herbs, *Bacillus* spp. 357 and 559 from the cecum of healthy chickens while *Bacillus* spp. 634 and 744 were originally obtained from the fresh faeces of healthy piglets.

The size of the inhibitory zone for the six selected isolates against the four indicator pathogens is shown in Table 1. An example of a plate diffusion assay indicating the inhibitory activity for *B. subtilis* MA139 against the four pathogens is shown in Fig. 1. All six *Bacillus* strains were most effective against the Gram-positive bacterium *Staphylococcus aureus*. However, all six strains of spore formers also showed high inhibitory activity against the three Gram-negative bacteria, *E. coli* K88, *E. coli* K99, and *S. typhimurium*. These bacteria are known as the main pathogens causing diarrhea in piglets (Asai et al. 2002; Fairbrother et al. 2005). The production of antimicrobials is considered one of the major mechanisms through which probiotics function and consequently is also one of the principle criteria for

**Table 1** The inhibition zone of six *Bacillus* isolates when incubated with four indicator pathogens ($n$=3)*

| Strains | Inhibitory zone (mm) | | | |
|---|---|---|---|---|
| | *E. coli* K88 | *E. coli* K99 | *Staphylococcus aureus* | *Salmonella typhimurium* |
| *Bacillus* spp. 23 | 29.8 ± 0.6 | 35.6 ± 0.7 | 46.8 ± 0.5 | 40.6 ± 0.8 |
| *B. subtilis* MA139 | 32.0 ± 1.0 | 35.9 ± 0.7 | 47.5 ± 0.5 | 42.5 ± 0.7 |
| *Bacillus* spp. 357 | 28.2 ± 0.4 | 35.0 ± 0.6 | 45.4 ± 0.8 | 40.8 ± 0.7 |
| *Bacillus* spp. 559 | 31.3 ± 0.9 | 33.6 ± 0.8 | 44.4 ± 1.1 | 38.3 ± 0.9 |
| *Bacillus* spp. 634 | 29.6 ± 0.7 | 31.5 ± 1.2 | 45.6 ± 0.6 | 38.6 ± 1.0 |
| *Bacillus* spp. 744 | 31.0 ± 1.0 | 33.6 ± 0.6 | 45.7 ± 0.8 | 38.4 ± 1.1 |

*Results are shown as mean ± s.d

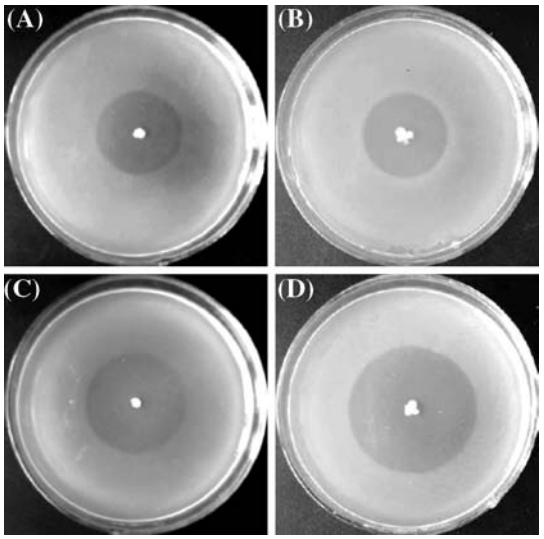


**Fig. 1** The inhibitory activity of *Bacillus subtilis* MA139 against four pathogenic indicator bacteria shown by the disc plate diffusion assay: (**A**) inhibition zone against *E. coli* K88; (**B**) inhibition zone against *E. coli* K99; (**C**) inhibition zone against *Salmonella typhimurium*; (**D**) inhibition zone against *Staphylococcus aureus*

strain selection when screening potential probiotics (Chang et al. 20001; Dunne et al. 2001; Hong et al. 2005). Therefore, the six *Bacillus* strains isolated were all thought to have potential to compete with pathogens and improve the balance of the microflora in the gastrointestinal tract. As a result, they were all subjected to further screening using simulated gut environments.

## Effect of simulated gastric fluid with pH 2.0 on spore viability

The spore viability of the six *Bacillus* strains when cultured in simulated gastric fluid with a pH of 2.0 is shown in Table 2. Five of the spores of six strains exhibited high resistance to simulated gastric fluid and there was no significant reduction in viability during the 3 h culture in simulated gastric fluid. The exception was *Bacillus* spp.744, which showed significantly reduced counts after 2 h ($P<0.05$). The finding that not all spores are equally resistant to simulated gastric fluid supports the findings of Hyronimus et al. (2000) and Duc le et al. (2004). The possible reason is the acid activation of spore germination and subsequent vegetative cells killed by simulated gastric fluids (Duc le et al. 2004; Hong et al. 2005).

Probiotics delivered through the feed system have to firstly survive during transit through the upper gastrointestinal tract (Huang and Adams 2004). Incubation of the spores in gastric juice with a pH of 2.0 for 3 h was an attempt to mimic the conditions that a probiotic would have to survive as it passes through the stomach of a pig. The fact that the viable counts of most of the six *Bacillus* strains did not decrease after 3 h of incubation suggests that a reasonable percentage of these *Bacillus* strains, if provided in feed, should survive passage through the harsh environment of the pig stomach.

## Effect of simulated small intestinal fluid on spore viability

Another barrier that probiotic bacteria must survive is passage through the small intestine (Huang and Adams 2004). The experimental results of Casula and Cutting (2002) suggest that *B. subtilis* germinates in the jejunum and ileum. The adverse conditions in the small intestine that probiotics must survive include the presence of bile salts and pancreatin (Floch et al. 1972).

The tolerance of the spores of six isolates to culture with 0.3% bile salts in simulated intestinal fluid

**Table 2** Effect of simulated gastric fluid on the viability of six *Bacillus* strains during a 3 h incubation *t* (*n*=3)*

| Strains | Viable counts (log CFU/ml) | | | |
|---|---|---|---|---|
| | 0 h | 1 h | 2 h | 3 h |
| *Bacillus* spp. 23 | 6.18 ± 0.04 | 6.29 ± 0.04 | 6.26 ± 0.04 | 6.20 ± 0.16 |
| *B. subtilis* MA139 | 6.76 ± 0.02 | 6.74 ± 0.01 | 6.69 ± 0.06 | 6.73 ± 0.00 |
| *Bacillus* spp. 357 | 6.69 ± 0.05 | 6.59 ± 0.07 | 6.60 ± 0.06 | 6.67 ± 0.04 |
| *Bacillus* spp. 559 | 6.60 ± 0.05 | 6.55 ± 0.06 | 6.56 ± 0.03 | 6.56 ± 0.13 |
| *Bacillus* spp. 634 | 6.61 ± 0.06 | 6.48 ± 0.04 | 6.60 ± 0.16 | 6.36 ± 0.14 |
| *Bacillus* spp. 744 | 7.27 ± 0.11[a] | 6.98 ± 0.05[a] | 6.61 ± 0.01[b] | 6.29 ± 0.20[b] |

*Results are shown as mean ± s.d

[a,b]Means in row with different superscripts are statistically different at $P<0.05$

for 5 days is shown in Table 3. On the first day of culture, none of the isolates grew in simulated intestinal fluid. However, on the second day, the simulated intestinal fluid inoculated with *B. subtilis* MA139 and *Bacillus* spp. 634 began to turn turbid while the other isolates and the control did not change. On the fifth day, the incubations of isolates *B. subtilis* MA139 and *Bacillus* spp. 634 were sampled and many spores had germinated. The tolerance of *B. subtilis* MA139 and *Bacillus* spp. 634 to simulated intestinal fluid with 0.3% bile salts suggested that it should be possible for their spores to germinate without being inhibited by the presence of bile salts in the small intestine. It could also be concluded that among the six strains the tolerance of spores to unfavorable conditions was different from each other and that the tolerance test to simulated gut environments was therefore an effective method with which to screen *Bacillus* for use as potential probiotics.

## Performance trials

The in vitro techniques for screening *Bacillus* as potential probiotics are far from the requirements in practice fofr farmers. *B. subtilis* MA 139 was selected for evaluation in the performance trial based on the fact that it had the largest inhibition zones against the four indicator bacteria (Table 1) and that it exhibited the best resistance to the unfavorable conditions within the simulated gut environments (Tables 2 and 3). Its' effects on the performance and faecal microflora of weanling pigs is shown in Tables 4 and 5. Over the 4-week feeding trial, all diets containing *Bacillus* supplements resulted in increased weight gain compared with the negative control ($P$=0.10). Feed conversion for piglets fed the *Bacillus* diet was significantly improved compared with the negative control ($P$=0.03). The performance of pigs fed spore-supplemented diets did not differ from that of pigs fed the antibiotic diet. These findings support earlier work with *Bacillus* species showing improvements in animal performance (Zani et al. 1998; Adami and Cavazzoni 1999; Kyriakis et al. 1999; Alexopoulos et al. 2004; Kritas and Morrison 2005). The diet containing $2.2\times10^5$ CFU/g of feed resulted in the most favorable growth performance but no significant difference was observed among the diets containing different concentrations of spores. The concentration of viable spores in the probiotic is considered crucial to obtain the desired effects (Zani et al. 1998) and inadequate concentrations of microorganisms could result to unsatisfactory results (Fuller 1986). However, in this study, *B. subtilis* MA139 was obtained by screening for its full resistance to unfavorable conditions within a simulated gut environment and there was expected to be no loss of viable spores during passage through the gastrointestinal tract. Therefore, the desired effect could be obtained even though the concentration of spores was in a low level.

Lactobacilli and *E. coli* in the faeces of the negative diet and the experimental diet with $2.2\times10^5$ CFU/g of feed were determined in faeces at three separate time

**Table 3** Determination of spore tolerance when *Bacillus* strains were cultured in simulated intestinal fluid with 0.3% bile for 5 days

| Strains | Tolerance |
|---|---|
| *Bacillus* spp. 23 | – |
| *B. subtilis* MA139 | + |
| *Bacillus* spp. 357 | – |
| *Bacillus* spp. 559 | – |
| *Bacillus* spp. 634 | + |
| *Bacillus* spp. 744 | – |

**Table 4** The effect of *B. subtilis* MA139 on the performance of weaned piglets during a 4 week feeding trial

| Treatments | Negative control (C) | *Bacillus* concentration (CFU/g of feed) (B) | | | Positive control (A) | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | | $2.2\times10^5$ | $2.2\times10^6$ | $2.2\times10^7$ | | C versus A | C versus B | A versus B |
| Average daily weight gain (g/day) | 458 ± 56 | 512 ± 66 | 505 ± 64 | 505 ± 35 | 485 ± 82 | 0.46 | 0.10 | 0.45 |
| Average daily feed intake (g/day) | 792 ± 107 | 824 ± 122 | 826 ± 85 | 823 ± 69 | 808 ± 116 | 0.79 | 0.51 | 0.73 |
| Feed conversion ratio | 1.74 ± 0.13 | 1.61 ± 0.11 | 1.64 ± 0.06 | 1.63 ± 0.11 | 1.68 ± 0.10 | 0.32 | 0.03 | 0.32 |

**Table 5** Faecal shedding of lactobacilli and *E. coli* shedding by piglets fed an unsupplemented diet or a diet containing $2.2\times10^5$ CFU/g *B. subtilis* ($n$=3)

| | | Control | *B. subtilis* MA139 | *P*-value |
|---|---|---|---|---|
| *Lactobacillus* spp. | d 0 | 7.24 ± 0.23 | 7.38 ± 0.11 | 0.69 |
| | d 14 | 6.77 ± 0.57 | 7.02 ± 0.32 | 0.37 |
| | d 28 | 6.66 ± 0.47 | 7.30 ± 0.26 | 0.02 |
| *E. coli* | d 0 | 3.62 ± 0.66 | 3.49 ± 0.85 | 0.85 |
| | d 14 | 3.82 ± 0.87 | 3.02 ± 0.23 | 0.05 |
| | d 28 | 3.39 ± 0.78 | 3.26 ± 0.47 | 0.80 |

points during the study (Table 5). *Lactobacillus* counts on day 28 indicated that piglets fed the diet containing $2.2 \times 10^5$ CFU/g of feed had significantly higher lactobacilli counts than piglets fed the negative control diet ($P$=0.02). Meanwhile, the addition of *B. subtilis* MA139 showed a tendency to decrease the counts of *E. coli* shed in the faeces on day 14 compared with the negative control ($P$=0.05). It has been previously reported that use of *Bacillus* strains as probiotics significantly increased the counts of gastrointestinal lactobacilli and decreased the counts of *E. coli* (Adami and Cavazzoni 1999; Hosoi et al. 1999).

The exact mechanism through which *Bacillus* strains may alter the type of microflora in the gastrointestinal tract remains uncertain. One explanation might be associated with the decreased oxidation–reduction potential cause by the germination of spores in the intestine, which has been shown to benefit the growth of lactobacilli (Vervaeke et al. 1973). In addition, *Bacillus* strains could produce some metabolites that inhibit pathogens, since some *Bacillus* species used in commercially available products have the ability to produce antimicrobials, such as aminocumaim A (Pinchuk et al. 2001) and bacteriocin (Zheng and Slavik 1999; Cladera-Olivera et al. 2004). However, further research is necessary to fully determine the exact mechanism through which the probiotic effects of *Bacillus* strains are achieved.

## Conclusions

The study isolated *B. subtilis* MA139 for probiotic use by initial co-culture with indicator pathogens, a disc plate diffusion assay and simulated gut environment tolerance tests. The results showed *B. subtilis* MA139 is a promising alternative to antibiotics for use as a feed additive in piglet diets.

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