Effect of dietary *Bacillus subtilis* on growth performance and serum biochemical and immune indexes in weaned piglets

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**ABSTRACT**

This study evaluated the effect of dietary *Bacillus subtilis* on growth performance of weaned piglets, and also the effects of dietary *B. subtilis* on serum biochemical parameters and serum growth hormone, cytokines and immunoglobulins levels after *Escherichia coli* challenge. A total of 24 weaned piglets were housed in single pens and fed the basal diet (NC) either with or without *B. subtilis* (BS) or chlortetracycline (PC) for a 14-day period. All piglets were orally inoculated with *E. coli* K88 on day 14, and the blood samples were collected at 6 hours post-challenge. The results showed that dietary BS increased average daily gain and reduced feed conversion rate compared to the NC group. In addition, piglets in the BS group had higher serum growth hormone levels, total protein concentrations and alkaline phosphatase activities than the NC group did. Moreover, dietary BS decreased concentrations of serum IFN-γ and TNF-α and increased concentrations of serum IL-10 compared to the NC group, and BS supplementation increased serum IgA and IgG concentrations compared to those of the NC group. In conclusion, dietary *B. subtilis* improved growth performance of weaned piglets and attenuated the serum immune response to *E. coli* challenge.

**Introduction**

Intensification of the swine industry has brought increased risks of both clinical and sub-clinical enteric disease. Weaned pigs can be easily infected with pathogenic *Escherichia coli* (Fairbrother et al. 2005), which can cause diarrhoea, high mortality, poor performance and other issues (Bhandari et al. 2008). The traditional way to solve these problems is to use antibiotics, but there are many negative effects of this approach. Many countries have taken measures to restrict the use of antibiotics in livestock. Thus, alternatives to antibiotics are needed.

The dosage of probiotics given can be increased to ensure good health and maintain growth performance of swine (Petti-grew 2006). *Bacillus* strains have some advantages over many common *Lactobacillus* products. *Bacillus* can produce spores and tolerate high temperatures and acid–base conditions, which is easy to store and can be dormant for a long time without any deleterious effect on viability (Mazza 1994; Hong et al. 2005). *Bacillus* strains have been authorized in the EU for using as a probiotic feed additive in sows and piglets and several other farm animal species (Scharek et al. 2007). As a kind of effective antibiotic substitute, *Bacillus subtilis* has been widely utilized in feed (Guo et al. 2006). *B. subtilis* has a significant preventive effect against diarrhoea caused by *Escherichia coli* in weaned piglets (Zani et al. 1998; Kyriakis et al. 1999). The protective effect of *B. subtilis* may be due to the produced antifungal compounds such as some peptides during its metabolism in the intestinal tract. Those peptides can inhibit pathogenic microorganisms effectively (Oscáriz and Pisabarro 2000) or exert efficacy by optimizing intestinal flora and reducing the redox potential in the intestinal tract (Guo et al. 2006).

Although many previous studies demonstrated the preventive effect of *B. subtilis* against diarrhoea caused by *E. coli* challenge in weaned piglets, an investigation on the systemic defence capacities that exist after *E. coli* challenge might also be important for understanding the beneficial effect of *B. subtilis* on post-weaning piglets. Thus, this work was conducted to determine whether the addition of *B. subtilis* to the diets of weaned piglets would enhance the growth performance of the piglets and protect against a pathogenic *E. coli* infection by improving serum growth hormone levels, biochemical indexes and the immune status, consequently providing a theoretical basis for the application of these strains in diets.

**Materials and methods**

All experimental protocols were approved by the Animal Care and Use Committee of the College of Animal Science and Veterinary Medicine, Tianjin Agricultural University.

**Bacterial strains**

The *Bacillus subtilis* strain used in this study was isolated from the faeces of a healthy piglet. *Bacillus subtilis* was cultured in Luria
Broth medium at 37°C for 24 h in an aerobic environment. A culture solution of the strain was centrifuged at 3,000 × g for 10 min at 4°C. Bacillus subtilis powder was acquired via treatment in a vacuum freeze-drying machine (Toffon, Shanghai, China), and there were 5 × 10^11 CFU/g of Bacillus in the freeze-dried powder. The bacterial concentration was determined by an ultraviolet spectrophotometer (Nano Drop, Thermo Fisher Scientific, Waltham, MA, USA) at an OD value of 550 nm.

The ETEC K88 strain (serotype O149:K91:K88ac), obtained from the China Institute of Veterinary Drug Control (Beijing, China), was grown in Luria Broth. The expanded culture of ETEC K88, containing approximately 1 × 10^10 CFU/mL, was further prepared for oral dosing as described by Zhang et al. (2010).

### Animals, diets and experimental design

A total of 24 crossbred healthy female piglets ([Yorkshire × Landrace] × Duroc) were reared by sows and weaned at 25 ± 2 days of age. The piglets (6.80 ± 0.65 kg) were allotted to 1 of 3 dietary treatments in a randomized complete block design according to their body weight. The dietary treatment groups were as follows: (1) a negative control group (NC) comprising piglets fed the basal diet; (2) a Bacillus subtilis (BS) group comprising piglets fed the basal diet supplemented with 10^2 CFU B. subtilis/kg feed; and (3) a positive control group (PC) comprising piglets fed the basal diet supplemented with 75 mg/kg chlortetracycline. There were 8 replicates in each group (4 gilts and 4 barrows) with 1 piglet per pen. The starting temperature of 24°C was adjusted weekly to reach a final temperature of 28°C. Each pen was equipped with a feeder and a nipple water to allow piglets free access to feed and drinking water. The corn-soybean basal diet (Table 1) was formulated to meet the National Research Council (NRC 2012) requirements for all nutrients.

At 08:00 on day 14, all the piglets were orally given a single dose of 10 mL PBS containing approximately 2 × 10^10 CFU of ETEC K88 using disposable pipets. After the E. coli challenge, the drinking water was cut off for 2 hours, and then the water supply was restored. Blood samples were collected from all piglets by jugular puncture into 10-mL plain tubes at 6 hours post challenge. Samples were allowed to clot for 30 min at room temperature and were then stored at 4°C overnight before harvesting serum by centrifugation at 3000 × g for 10 min at 4°C.

### Experimental observations and measurements

All piglets were individually weighed at weaning (day 0) and at day 14. Feed intake was recorded daily, and the residual feed was measured at the end of the trial. The average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated for each piglet.

The following serum biochemical parameters were determined using a HITACHI 7020-automatic biochemical analyser (Hitachi Ltd., Tokyo, Japan) and commercial kits according to the protocols provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China): total protein (TP), albumin (ALB), globulin (GLB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and urinary nitrogen (UN).

Quantification of growth hormone (GH), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), immunoglobulin A (IgA) and immunoglobulin G (IgG) in serum samples were assayed in duplicate using commercial porcine ELISA kits according to the protocols provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results were expressed as nanograms per millilitre for GH and cytokines and as milligrams per millilitre for Ig A and Ig G based on standard curves.

### Statistical analysis

Data were analyzed as a completely randomized block design by an ANOVA using the GLM procedure in SAS v. 9.2 (SAS Institute Inc., Cary, NC, 2002). The model included the treatment effect, and the individual piglet represented the experimental unit for all the parameters. Treatment comparisons were done using a Tukey’s honest significant difference test for multiple testing. Probability values of 0.05 were considered to be significant, and a treatment effect trend was noted for values of P ≤ 0.10.

### Results

#### Growth performance

Table 2 shows the body weight, ADFI, ADG, and FCR data computed for the piglets pre-challenge. Supplementation with B. subtilis and chlortetracycline markedly increased the average daily gain (P = 0.009) and reduced the feed conversion
Table 2. Effect of dietary *B. subtilis* on growth performance of weaned piglets1.

| Items  | NC    | BS    | PC    | SEM  | P-value |
|--------|-------|-------|-------|------|---------|
| BW, kg |       |       |       |      |         |
| Day 0  | 6.78  | 6.77  | 6.77  | 0.07 | 0.996   |
| ADG, g/d | 252a  | 285a  | 293a  | 6    | 0.009   |
| FCR    | 1.56a | 1.43b | 1.36b | 0.02 | 0.884   |
| ADFI, g/d | 394  | 408   | 400   | 11   | 0.001   |
| P-value |       |       |       |      |         |

NC = basal diet without supplementation; BS = NC + 10^7 CFU/kg *Bacillus subtilis*; PC = NC + 75 mg/kg Chlortetracycline; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.

Table 3. Effect of dietary *B. subtilis* on serum biochemical indexes of weaned piglets at 6 hours post *E. coli* challenge1.

| Items  | NC    | BS    | PC    | SEM  | P-value |
|--------|-------|-------|-------|------|---------|
| TP, g/L |       |       |       |      |         |
| ALB, g/L | 30.17 | 29.66 | 30.69 | 0.64 | 0.821   |
| ALT, U/L | 37.5  | 38.18 | 35.42 | 0.68 | 0.235   |
| ALP, U/L | 233b  | 261a  | 255a  | 4    | 0.017   |
| UN, mmol/L | 2.35  | 2.14  | 2.05  | 0.06 | 0.139   |
| P-value |       |       |       |      |         |

NC = basal diet without supplementation; BS = NC + 10^7 CFU/kg *Bacillus subtilis*; PC = NC + 75 mg/kg Chlortetracycline; TP = total protein; ALB = albumin; GLB = globulin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; UN = urinary nitrogen.

Table 4. Effects of dietary *B. subtilis* on serum concentration of cytokines and immunoglobulins in weaned piglets at 6 hours post *E. coli* challenge1.

| Items  | NC    | BS    | PC    | SEM  | P-value |
|--------|-------|-------|-------|------|---------|
| IL-6, ng/mL | 1.92  | 1.74  | 1.83  | 0.03 | 0.097   |
| IL-10, ng/mL | 19.35b | 22.67a | 21.46a | 0.45 | 0.004   |
| TNF-α, ng/mL | 135.68a | 96.75c | 110.23b | 3.41 | <0.001  |
| IgA, mg/mL | 4.34b  | 5.36a  | 4.65b  | 0.40 | <0.001  |
| IgG, mg/mL | 16.52b | 18.39b | 16.06b | 0.11 | 0.018   |
| P-value |       |       |       |      |         |

NC = basal diet without supplementation; BS = NC + 10^7 CFU/kg *Bacillus subtilis*; PC = NC + 75 mg/kg Chlortetracycline; IL-6 = interleukin-6; IL-10 = interleukin-10; IFN-γ = interferon gamma; TNF-α = tumour necrosis factor alpha; IgA = immunoglobulin A; IgG = immunoglobulin G.

Serum growth hormone levels and biochemical parameters

The effect of *B. subtilis* supplementation on the serum growth hormone concentration is presented in Figure 1. The GH level in the NC group was lower than that in the BS and PC groups (P = 0.020). However, there was no significant difference between the BS and PC groups (P > 0.05). As shown in Table 2, piglets in the BS and PC groups had higher serum TP concentrations (P < 0.001) and ALP activity (P = 0.017) than those in the NC group, while no significant differences in serum biochemical parameters between the BS and PC groups were observed (P > 0.05).

Serum cytokines and immunoglobulins

Table 4 shows the effect of *B. subtilis* supplementation on serum cytokines and immunoglobulins post *E. coli* challenge. Dietary *B. subtilis* and chlortetracycline markedly decreased concentrations of serum IFN-γ and TNF-α (P = 0.004 and P < 0.001, respectively) and increased concentrations of serum IL-10 (P < 0.001) compared to values in the NC group, and...
piglets in the BS group had lower concentrations of serum IFN-γ and TNF-α than those in the PC group ($P = 0.004$ and $P < 0.001$, respectively). In addition, *B. subtilis* supplementation markedly increased serum IgA and IgG concentrations compared to those in the NC and PC groups ($P < 0.001$ and $P = 0.018$, respectively). Moreover, supplementation with *B. subtilis* tended to reduce the concentration of IL-6 compared to that observed in the NC group ($P = 0.097$).

**Discussion**

Piglets suffer from weaning stress, which leads to lowered immune function, enhances susceptibility to easy pathogen infection and can thus harm the organism’s health. Previous research has shown that *Bacillus* can stimulate the function of the immune system, reducing weaning stress and improving growth performance (Lee et al. 2014). In our study, *Bacillus subtilis* supplementation to diets significantly increased ADG and reduced FCR of piglets, which is consistent with previous observations (Kyriakis et al. 1999; Jørgensen et al. 2016). Growth hormone (GH) is one of the most important hormones in controlling the overall growth of animals and functions to promote protein deposition and the growth of bone and muscle (Pell and Bates 1990). Our study showed that supplementary *Bacillus subtilis* could significantly enhance the GH concentration in the serum, which was consistent with the increased growth rate of the piglets. During the post-weaning period, the digestive organs of piglets are still not completely developed, and the ability to digest nutrients is low. It has been reported that some digestive enzymes produced by *Bacillus* could promote the absorption of nutrition and improve feed conversion in animals (Latorre et al. 2016). Previous studies have shown that *Bacillus* can significantly improve lipase activity in the jejunum of piglets and reduce taurocholic acid depolymerase activity of bacteria that are contained in the jejunal digesta, thereby promoting the digestion and absorption of fat (Jadamus et al. 2001). On the other hand, previous studies have also shown that dietary supplementation with *Bacillus subtilis* can increase the quantity of *Lactobacilli* in the gastrointestinal tract of pigs, thereby reducing proliferation of *Escherichia coli* and promoting microbial flora balance (Kornegay and Risley 1996; Turner et al. 2001). Therefore, supplementation with *Bacillus subtilis* may have positive effect on the growth performance of weaned piglets that is probably due to improved endocrine hormone levels and enhanced health status.

Changes in serum biochemical indexes are a result of changes in tissue cell permeability and changes in the metabolism function in the body. Serum protein is an important sign of protein anabolism in the body, and the total protein (TP) and albumin content reflect the body’s absorption and metabolism of protein (Coma et al. 1995). An increase in the protein content of the serum is conducive to improving the metabolism levels and immunity of animals, promoting protein synthesis and increasing nitrogen deposition (Kanjapraputhipong 1998). Our results showed that piglets fed the diet supplemented with *Bacillus subtilis* experienced an increase in the concentration of total protein in serum, which is consistent with previous studies. Studies have shown that supplementation with 400 mg/kg *Bacillus subtilis* in the diet can increase the digestibility of crude protein in piglets and improve the growth performance of pigs (Jørgensen et al. 2016), which might be due to the ability of dietary *Bacillus subtilis* to improve protease secretion in the digestive tract of pigs. In addition, our study also showed that supplementation with *Bacillus subtilis* numerically decreased the serum UN level, which was consistent with the trend of TP results in the experiment. The serum UN concentration to some extent reflects the condition of protein metabolism and amino acid utilization in the whole body (Chen et al. 2017). Therefore, *Bacillus subtilis* can increase the digestibility of crude protein, increase serum protein levels, and promote the growth of pigs. Colonization by *E. coli* K88 and attachment of these bacteria to the ileal mucosa have been found to stimulate the host’s innate immune response and lead to inflammation (Daudelin et al. 2011). The endotoxin dephosphorylating activity of ALP may reflect an essential physiological function of the enzyme, and the induction of ALP activity during inflammation and cholestasis is in accordance with a role for ALP in host defence (Poelstra et al. 1997). The present study showed that the activity of serum ALP in piglets in the *Bacillus subtilis* group increased significantly, which may suggest that *Bacillus subtilis* plays a direct role in reducing the harm done by pathogenic *E. coli* K88. However, the BS group had no significant increases in ALB, GLB, AST or ALT compared to the control animals, which indicated that *Bacillus subtilis* supplementation had no significant effect on liver function of piglets infected by a pathogenic infection.

Enterotoxigenic *Escherichia coli* infection is associated with the production of pro-inflammatory mediators in the intestine, including IL-6 and tumour necrosis factor alpha (TNF-α) (Ji et al. 2013). TNF-α can cause tissue injury and shock (Soni and Adebiyi 2017). *Bacillus amyloliquefaciens* could effectively down-regulate IL-6 and TNF-α mRNA production in differentiated IPEC-1 cells after ETEC treatment (Ji et al. 2013). The immune response to pathogen exposure includes the release of a wide variety of vasoactive and inflammatory mediators that can alter vascular resistance and haemodynamics, and/or promote cell death. Pro-inflammatory cytokines, including interferon-gamma (IFN-γ) and TNF-α, which are elevated during sepsis, activate cellular signal transduction pathways that promote apoptotic cell death (Soni and Adebiyi 2017). Compared to the results in the control group, the production of the pro-inflammatory cytokines IFN-γ and TNF-α in the serum were significantly reduced by supplementation with *B. subtilis* and antibiotics. IL-6 is an important inflammatory mediator and is also an important inflammatory cytokine. As an anti-inflammatory factor, IL-10 mainly affects mononuclear phagocyte, inhibits adhesion of these cells, and activates production of a variety of cytokines, such as by inhibiting the pro-inflammatory cytokines TNF, IL-6 and so on (Knolle et al. 1997; Buer et al. 1998). In our study, the level of serum IL-6 in the BS group tended to be lower than that in the NC group. However, the expression of the anti-inflammatory cytokine IL-10 increased significantly in the BS group compared to the NC group. *Bacillus subtilis* can increase the expression of IL-6 in the small intestine of newborn piglets, increase the expression of defensin-2 in the duodenum of pigs (Deng et al. 2013) and enhance oral mucosal immune function.
(Amuguni et al. 2012). Therefore, Bacillus subtilis is beneficial in improving the anti-infection abilities of piglets to combat bacterial agents.

During suckling, piglets ingest the sow’s milk, which contains IgA that is absorbed into the mucus covering the villous surfaces and prevents E. coli and other organisms from attaching to the villi (Zhang et al. 2010). The relationship between maternal IgA and the piglet IgA level is causal, thus the levels rapidly decline and bacteria damage the villi because no more maternal IgA is available after weaning (Zhang et al. 2010). IgA oligosaccharides partially inhibit the adherence of the K88 strain to porcine intestinal mucins. If the organisms are unable to attach, they are unable to cause disease (Ramos-Clamont et al. 2007; Zhang et al. 2010). Partial spores could enter into lymphoid tissues of the mesentery lymph after the spores were taken orally and could promote significant improvements in levels of anti-spore IgG and secretory surfaces and prevents improving the anti-infection abilities of piglets to combat bacterial agents (Duc et al. 2003).

Bacillus subtilis could promote the formation of animal gut associated lymphoid tissue in a high degree of ‘immunization readiness status’ and accelerate the development of immune organs. Both humoural and cellular defences of invertebrates could be increased by exposure to Bacillus species (Hong et al. 2005). Moreover, Enterococcus faecium and Bacillus subtilis could be used instead of some antibiotics in piglets’ diets, and these two probiotics could increase the growth performance, improve immunity and act as alternatives to antibiotics in the diets of piglets (Ramos-Clamont et al. 2006). In the present study, IgA and IgG levels of piglets in the BS group were significantly higher than those of piglets in the control and antibiotic groups. The increased concentrations of serum IgA and IgG observed in the present experiment in piglets fed a diet supplemented with Bacillus subtilis is reflective of improved immune function, which is consistent with prior findings (Lee et al. 2014).

Conclusions

In conclusion, the current study showed that dietary supplementation with Bacillus subtilis enhanced systemic resilience against E. coli challenge via modulation of growth performance, serum growth hormone levels, serum biochemical indexes and the serum immune status. Our results also suggest that probiotic Bacillus subtilis is worthy of further investigation as a good candidate to improve the health status of piglets and as a growth promoting agent during the post-weaning period.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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