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Effects of Feeding a Combination of Probiotics Containing *Lactobacillus plantarum* and *Bacillus Subtilis* on Immune Response and Diarrhea Incidence in Post-weaning Piglets

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Abstract : A study investigated the effects of a mixture of *Lactobacillus plantarum* (*L. plantarum*) and *Bacillus subtilis* (*B. subtilis*) on diarrhea incidence, immune response, and fecal microflora counts in post-weaning piglets. One hundred 28-day-old piglets were randomly assigned to five treatment groups: negative control (NC), free of antibiotics; positive control (PC), 0.03% chlortetracycline; LB 1, a mixture of *L. plantarum* and *B. subtilis* (LB) 0.5 kg/ton feed; LB 2, LB 1.0 kg/ton feed; and LB 3, LB 2.0 kg/ton feed. Diarrhea scores for LB 2 and LB 3 from the 2nd week were significantly reduced compared to NC ($P < 0.05$). In terms of the level of IgG and IFN-γ, all treatment groups were significantly higher than NC ($P < 0.05$), and the IgG level of LB 3 was significantly higher than that of PC ($P < 0.05$). Furthermore, fecal lactic acid bacteria (LAB) counts for LB 2 and LB 3 were significantly higher than those of NC and PC ($P < 0.05$). In addition, fecal *Enterobacteriaceae* (ENT) counts for PC, LB 2 and LB 3 were significantly lower than those of NC ($P < 0.05$). Based on the results from this study, it was concluded that a combination of *L. plantarum* and *B. subtilis* strains could be used as potential alternatives to antibiotics to prevent diarrhea incidence in piglets.

Key words **:** *Bacillus subtilis*, *Lactobacillus plantarum*, fecal microflora, diarrhea scores, piglets.

Introduction

Globally, antibiotics have been used in the swine industry for growth promotion, and the prevention and treatment of diseases for over fifty years (13). Despite the many advantages of antibiotics, the repeated use of antibiotics in swine feed has caused many problems such as the emergence of antibiotic-resistant bacteria, antibiotic residues in edible animal products, and disturbance to normal intestinal microflora (1,25). Due to these antibiotics problems, many countries have banned or rigorously limited their use in the animal industry. With the tendency to ban the use of antibiotics, the swine industry cannot but become interested in alternatives to antibiotics for growth promotion and maintaining health under commercial conditions.

Less than two decades later, probiotics are being used as an effective alternative to antibiotics in animal nutrition (7,9). Probiotics are a group of non-pathogenic organisms that are known to have positive effects on animal health (19). Many studies have reported the beneficial effects of probiotics on livestock in terms of growth performance, nutrient retention, gut health and intestinal microflora (4,20).

Recently, spore-forming *Bacillus* spp. have been regarded as a suitable probiotic due to the resistance of their spores to poor surroundings and long-term storage at room temperature (20). *Bacillus* species are able to synthesize antibiotics, proteases, amylases, amino acids, and other metabolites and exhibit immunomodulating activity, which accounts for their use in some therapeutic- and prophylactic-purpose commercial probiotic preparations (11). *Bacillus subtilis* (*B. subtilis*) is a Gram-positive, non-pathogenic, spore-forming organism normally found in the soil, and the robustness of its spores is thought to enable passage across the gastric barrier, where many spores germinate in the small intestine and populate (28). Additionally, *Lactobacillus* spp. are one of the most beneficial probiotics and are tolerant to bile salts and low pH (27). *Lactobacillus plantarum* (*L. plantarum*) has antagonistic potential against intestinal pathogens due to the production of lactic acid and/or bactericidal compounds (24).

However, information on the combination effects of *B.*

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subtilis and *L. plantarum* on gut microflora and the prevention of diarrhea in piglets is still limited. Thus, the objective of this study was to evaluate the efficacy of a mixture of *Lactobacillus plantarum* SY-99 and *Bacillus subtilis* SJ-61 spores on the immune response, fecal microflora and diarrhea incidence in piglets.

Materials and Methods

Materials

The probiotic mixture (PM) (Lactorin®, Dae Han New Pharm, Seoul, Korea) contained *L. plantarum* (SY-99) 2.4×10^9 CFU/kg and *B. subtilis* (SJ-61) 2.2×10^9 CFU/kg. *L. plantarum* (SY-99) was isolated from salted and fermented seafood, and *B. subtilis* (SJ-61) was isolated from fermented soya beans.

Animals

One hundred 28-day-old crossbred piglets (Yorkshire × Landrace × Duroc) (average body weight, 6.76 ± 0.45 kg) were purchased from a commercial supplier (Kayainte Co., Ltd., Sacheon, Korea). The piglets were kept in raised slatted floor pens (1.2 × 1.6 m) at 26 ± 1°C and the humidity was 65 ± 5%. Water and feed were offered *ad libitum* throughout the experimental period. This experiment was carried out at the pig farm at the Livestock Farm of Gyeongsang National University, Chinju, Korea. The protocol used for this experiment complied with the guidelines of the Ethical Committee of Gyeongsang National University.

Study design

The piglets were randomly assigned to one of five treatment groups. The basal diet was formulated to meet the nutrient requirements of piglets according to the NRC (15). The five treatment groups were NC (negative control; basal diet without antibiotics), PC (positive control; basal diet + chlortetracycline 0.3 g/kg), LB 1 (basal diet + 0.5 g/kg PM), LB 2 (basal diet + 1.0 g/kg PM), and LB 3 (basal diet + 2.0 g/kg PM). The experiment was carried out for four weeks and the initial and final body weights were measured. At the end of the experiment, a 5-ml blood sample was collected from the anterior vena cava of piglets into a non-heparinized vacuum tube (BD Vacutainer®, BD, NJ, USA). After all the collected blood samples were centrifuged (20 min, 2,000 × g at 4°C), serum samples were stored at −80°C.

Analysis of serum immunoglobulin G (IgG) and interferon-γ (IFN-γ)

The concentration of serum IgG and IFN-γ was determined according to the manufacturer's protocol using a porcine-specific ELISA kit (IgG Pig ELISA Core Kit; KOMA Biotech, Seoul, Korea; Porcine IFN-gamma ELISA Kit; Ray Biotech, Norcross, GA, USA). All assays were performed in triplicate.

Diarrhea scores and feces sampling

The diarrhea score of each piglet was recorded every week. The severity of diarrhea was noted by visually scoring the consistency of the feces on a standardized scale of 0-3 as described by Cox *et al*. (5): 0, no diarrhea (normal feces); 1, slight (pasty feces); 2, moderate (semi-liquid feces); and 3, severe diarrhea (watery feces). Fecal samples were collected directly around the anus of each piglet and were then stored at −20°C until further analyses for micro-floral counts.

Fecal LAB and ENT counts

One gram of feces was diluted in sterile 2.25% peptone water and homogenized to stand at room temperature for 1 h. The samples were diluted for further tenfold series with 2.25% peptone water. The diluted samples were shaken using a vortex mixer and spread onto an agar plate. Enumerations of LAB were performed on MRS agar (LAB M, Bury, UK) and incubated in an anaerobic jar at 30°C for 48 h. ENT enumerations were performed on eosin methylene blue agar (Merck, Darmstadt, Germany) and incubated under anaerobic conditions at 37°C for 24 h. The number of colony-forming units (CFUs) is expressed as $\log_{10}$ CFU per g.

Statistical analyses

Values are presented as the mean ± standard deviation (SD). All data were analyzed using one-way analysis of variance (ANOVA) (SAS Institute, NC, USA) followed by a two-tailed Student's *t*-test when the ANOVA yielded statistically significant differences ($P < 0.05$). All statistical analyses were performed using the Stat View J-5.0 program (SAS Institute Inc., Cary, NC, USA). All data were expressed as the mean ± standard deviation (SD).

Table 1. Change in body weight for various experimental groups

Items	Treatments*				
	NC	PC	LB 1	LB 2	LB 3
Initial body weight (kg)	6.8 ± 0.57[a]	6.8 ± 0.72[a]	6.8 ± 0.66[a]	6.8 ± 0.45[a]	6.7 ± 0.67[a]
Final body weight (kg)	16.1 ± 1.05[a]	18.8 ± 1.14[b]	17.6 ± 1.27[a]	18.5 ± 1.22[b]	20.6 ± 1.18[b]
Change (kg)	9.3 ± 0.75[a]	12.0 ± 0.87[bc]	10.8 ± 0.89[a]	11.7 ± 0.65[b]	13.1 ± 1.06[c]

Data are expressed as mean ± SD.
*NC, negative control; PC, positive control; LB 1, NC + 1 g/kg PM (probiotic mixture of *L. plantarum* and *B. subtilis*); LB 2, NC + 2 g/kg PM; LB 3, NC + 4 g/kg PM.
[a-c]Figures with different superscripts in each row differ significantly ($P < 0.05$).

Results

Change of body weight and diarrhea scores

The final body weight and change of body weight in PC, LB 2 and LB 3 were significantly higher than those of NC ($P < 0.05$) while there were no significant differences between PC, and LB 2 and LB 3 (Table 1).

Diarrhea scores for all experimental groups are shown in Fig 1. Diarrhea scores for LB 2 and LB 1 were significantly lower than those of NC throughout the experimental period ($P < 0.05$). From the 2nd week, the diarrhea scores of LB 3 were significantly lower than that of PC ($P < 0.05$).

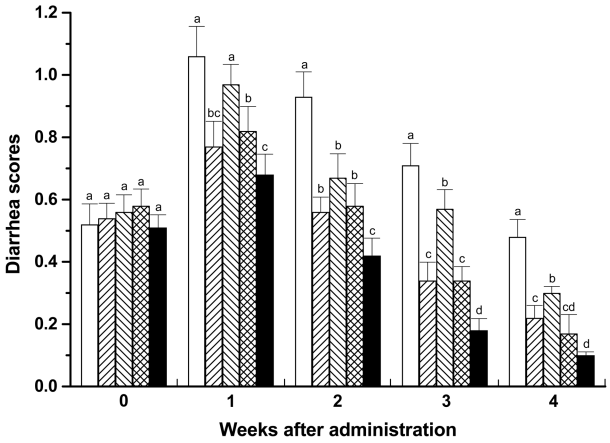

Fig 1. Diarrhea scores in piglets administered with different dietary treatments. NC (□), negative control; PC (▨), positive control; LB 1 (▧), NC + 1 g/kg PM; LB 2 (▩), NC + 2 g/kg PM; LB 3 (■), NC + 4 g/kg PM.
[a-d]Figs with different superscripts in each row differ significantly ($P < 0.05$).

Analysis of IgG and IFN-γ

Table 2 shows the serum levels of IgG and IFN-γ for all experimental groups. Serum IgG levels in all groups treated with PM increased significantly compared to that of NC ($P < 0.05$). Additionally, the serum IgG level for LB 3 was significantly higher than that of PC ($P < 0.05$). The IFN-γ serum level for PC and all PM treatment groups increased significantly compared with NC ($P < 0.05$). In addition, the IFN-γ serum level for LB 2 and LB 3 was slightly higher than that for PC, but there was no significant difference.

Fecal microfloral population

Fecal LAB counts for PC, LB 2 and LB 3 were significantly higher than those of NC ($P < 0.05$) (Table 3). Additionally, fecal LAB counts for LB 2 and LB 3 were significantly higher than those of PC ($P < 0.05$). The fecal ENT counts for PC, LB 2 and LB 3 decreased significantly compared with NC ($P < 0.05$).

Discussion

In this experiment, the supplementation of PM improved the final body weight in post-weaning piglets. Better growth performances in animals fed with PM could be due to the organic acids, bacteriocins, ethanol, hydrogen peroxide or vitamins present in the metabolites of *L. plantarum* (2,6), or due to hydrolytic enzymes produced by *B. subtilis* (18). Furthermore, supplementation of PM could have a greater effect

Table 2. Total serum IgG and IFN-γ levels in piglets fed with different dietary treatments

Items	Treatments*				
	NC	PC	LB 1	LB 2	LB 3
IgG (mg/ml)	17.7 ± 1.24[a]	19.6 ± 1.57[b]	20.1 ± 1.71[b]	22.7 ± 1.63[bc]	24.4 ± 2.04[c]
IFN-γ (pg/ml)	61.9 ± 4.18[a]	72.1 ± 4.23[b]	71.5 ± 5.32[b]	73.1 ± 4.89[b]	76.5 ± 5.03[b]

Data are expressed as mean ± SD.
IgG, immunoglobulin G; IFN-γ, interferon-gamma.
*NC, negative control; PC, positive control; LB 1, NC + 1 g/kg PM (probiotic mixture of *L. plantarum* and *B. subtilis*); LB 2, NC + 2 g/kg PM; LB 3, NC + 4 g/kg PM.
[a-c]Figs with different superscripts in each row differ significantly ($P < 0.05$).

Table 3. Fecal LAB and ENT populations in piglets fed with different dietary treatments

Items	Treatments*				
	NC	PC	LB 1	LB 2	LB 3
LAB	6.48 ± 0.13[a]	6.73 ± 0.11[b]	6.65 ± 0.14[ab]	7.28 ± 0.08[c]	7.36 ± 0.14[c]
ENT	6.26 ± 0.17[a]	5.87 ± 0.12[b]	6.21 ± 0.18[a]	5.92 ± 0.15[b]	5.71 ± 0.09[b]

Data are expressed as mean ± SD.
LAB, lactic acid bacteria; ENT, *Enterobacteriaceae*.
*NC, negative control; PC, positive control; LB 1, NC + 1 g/kg PM (probiotic mixture of *L. plantarum* and *B. subtilis*); LB 2, NC + 2 g/kg PM; LB 3, NC + 4 g/kg PM.
[a-c]Figs with different superscripts in each row differ significantly ($P < 0.05$).

on the increase in body weight than either supplement alone as seen from the results of a study by Hosoi *et al*. (8).

In a previous study (3), IgG was not affected by the treatment of dietary complex probiotics (*Lactobacillus acidophilus*, *Saccharomyces cerevisae* and *Bacillus subtilis*) on growing pigs for 42 days. On the contrary, the administration of mixtures of *Lactobacillus spp.* and *Streptococcus spp.* increased the immune function in piglets (26). In addition, the concentration of IFN-γ in serum increased significantly compared with the control after administration of *Lactobacillus fermentum* I5007 2.0×10^9 CFU per day to piglets for 10 days ($P < 0.05$) (29). Based on the results of this study, the increase in IgG and IFN-γ was similar to the results of previous studies (26,29). It has been reported that IFN-γ promotes immune responses at the initiation of several bacterial infections; i.e., macrophage activation, Th1-cell development, Th2 response inhibition and epithelial defense, including the induction of antimicrobial defenses (21). In addition, it has been reported that bacteria-specific serum IgG plays a critical role in the eradication of pathogenic bacteria (22).

In a previous study on the dietary effects of the metabolites of *L. plantarum* on the diarrhea scores of piglets, the administration of 0.3% metabolites of *L. plantarum* resulted in diarrhea scores that were considerably lower than 0.2 on the 10th day after treatment (25). In addition, the diarrhea scores of piglets in another previous study were 0.9 after 20% *Bacillus subtilis*-fermented soya beans were administered to piglets challenged with enterotoxigenic *Escherichia coli* K88 for four weeks (10). Numerous studies on the mechanisms of probiotic action in intestinal disease have indicated that the beneficial effects of probiotics may be either direct or indirect through modification of the local microbiota, epithelial barrier function, intestinal inflammation, or the immune system (17). Taking into consideration the administration dosage, the effect of PM on piglet diarrhea was similar to that of *L. plantarum* metabolites and greater than that of *Bacillus subtilis*-fermented soya beans.

Many researchers have reported that the supplementation of *L. plantarum* metabolites and *B. subtilis* in piglets reduced the fecal ENT population because of antibacterial components in the metabolites, and the competition and stimulation of effective microorganisms (14). Moreover, *L. plantarum* and *B. subtilis* in all treatment groups in this study could compete with ENT pathogens for surface area and nutrients in the gastrointestinal tract and increase the available energy to the host (14,16). However, Kirchgessner *et al*. (12) did not find any effect from *B. cereus* on diarrhea frequency in post-weaned piglets. In the above researches, the differing effects of *Lactobacillus* and *Bacillus* supplements on piglet diarrhea could have been as a result of the supplement dose being too high or low, or combinations of different *Bacillus* and *Lactobacillus* strains may have dissimilar effects.

In this study, an immune response, lower diarrhea scores, positive development of LAB population, and reduction of ENT were observed in piglets administered with a combination of *L. plantarum* and *B. subtilis* strains. It can be concluded that the combination of *L. plantarum* and *B. subtilis* strains are potential alternatives to antibiotics that could be used to promote growth and prevent enteritis in piglets.

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이유자돈에 대한 *Lactobacillus plantarum*과 *Bacillus subtilis* 합제 투여에 따른 면역반응과 설사발생에 미치는 효과

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요 약 : 본 연구는 *Lactobacillus plantarum* (*L. plantarum*)과 *Bacillus subtilis* (*B. subtilis*)의 합제 (LB)를 이유자돈에 경구 투여하여, 면역반응, 설사발생 그리고 분변 중 균수변화 등에 미치는 효과를 평가하기 위해 수행하였다. 28일령의 이유자돈 100두를 대상으로 군 당 20두씩 5개 군 (NC, 항생제와 LB 무투여; PC, 0.03% chlortetracycline 투여; LB 1, LB 0.5 kg/ton feed; LB 2, LB 1.0 kg/ton feed; LB 3, LB 2.0 kg/ton feed)으로 나누어, 4주 동안 LB를 투여하면서, 1주일 간격으로 분변지수를 산출하여 균별로 기록하였으며, 투여 종료 후, 혈액 및 분변을 채취하여, 혈액시료로부터 IgG와 IFN-γ를 분석하였고, 분변시료로부터 lactic acid bacteria (LAB)와 Enterobacteriaceae (ENT)의 수를 확인하였다. LB 투약 2주째부터, LB 2와 LB 3의 설사지수는 NC에 비해 통계적으로 유의성 있게 감소하였으며 ($P < 0.05$), IgG와 IFN-γ의 농도도 NC에 비해 통계적으로 유의성 있게 증가하였다($P < 0.05$). 또한, LB 2와 LB 3의 LAB와 ENT 수는 NC과 PC에 비해 통계적으로 유의성 있게 변화하는 결과를 나타내었다($P < 0.05$). 이상의 결과로부터, LB는 강력한 항생제 대체제로서 자돈 설사예방에 사용될 수 있을 것으로 사료된다.

주요어 : *Lactobacillus plantarum, Bacillus subtilis*, 분변 세균총, 설사지수, 자돈.