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Research Article

Effect of *Bacillus Megaterium 2333*, *Lactobacillus Casei 76* and Their Combination as a Probiotic Supplement Feed on the Growth Performance of Piglets

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Abstract

To feed piglets during the weaning period is critical in swine production farming. Probiotics could be alternative to antibiotics to reduce suckling stress and improve growth performance. The aim of this study was to investigate the effect of supplemental feed manufactured by *Bacillus megaterium 2333*, *Lactobacillus casei 76* and their combination on the growth performance of suckling piglets. First, the endurance to harsh environment and adhesion ability were tested to evaluate the possibility of probiotic use for these two bacteria. Then, supplemental feed was manufactured by addition of chosen bacteria. Feed composition was chemically analyzed. Four groups of piglets, namely Control, *Bacillus megaterium* 2333, *Lactobacillus casei 76* and their combination group were created to compare the effect. The result showed that supplemental feed manufactured by probiotic addition exerted beneficial effect on the growth performance of suckling and weaned piglets regardless of nutritional value of feed. Incidence of diarrhea and the number of pathogen such as *E. coli* and *Salmonella* were also lower in probiotic group. Especially, combination use of *Bacillus megaterium 2333* and *Lactobacillus casei 76* showed the best result of lowest feed conversion ratio. The result of present study indicated that the supplemental feed manufactured by the combination of *Bacillus megaterium 2333* and *Lactobacillus casei 76* was beneficial to rearing piglets.

Keywords

Probiotics, Piglet, Supplemental Feed, *Bacillus*, *Lactobacillus*

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1. Introduction

Weaning is one of the main challenges that piglets face throughout their entire lives. During the weaning period, piglets experience feed change, which may induce dysfunctions of intestinal and immune system. Weaning stress may negatively change the gut microbial ecosystem of piglets; consequently induce serious diseases such as diarrhea due to pathogen susceptibility [1]. Therefore, to control feed composition effectively for piglets is critical in swine production industry for healthy pig growth. The use of antibiotic feed additives is a frequent and effective method to reduce disease occurrence and increase pig production. However, the overuse or prolonged usage of antibiotics could cause unwanted problems such as the emergency of antibiotic resistant pathogens and negative change of gut microbiota. The concern to the misuse of antibiotics inspires to find alternatives in livestock farming [2].

Probiotics are viable microbial feed supplement which are beneficial to the host animal by improving its microbial balance. After the proposal of first concept of probiotics, a large number of researches have been conducted to apply them to livestock farming practice and positive results have been published. Probiotics could enhance intestinal health via various mechanisms such as stimulation of immunity, inhibition of pathogens, induction of antibiotic substances and competition for limited nutrients [3, 4]. Among various bacteria used as probiotics, *Bacillus sp* are frequent used ones, mainly because its spore-forming ability which is adventurous for long term survival in harsh environment. Previous studies demonstrated that *Bacillus sp* could modify intestinal microbiota and increase pig production [5, 6].

Lactic acid bacteria are another commonly used probiotics in swine production industry. Lactic acid could produce a huge amount organic acid which are beneficial to the growth of adventurous bacteria and inhibit pathogens. In addition, lactic acid bacteria produce several enzymes and vitamins into the intestinal lumen [7].

Although a large volume of literature has been published in terms of probiotic supplemental feed for suckling piglets, a lack of data is currently available to investigate the combination effect of *Bacillus megaterium* and *Lactobacillus casei*. Therefore, the present study was conducted to investigate the effect of probiotic supplemental feed composed of *Bacillus megaterium* and *Lactobacillus casei* on piglets.

2. Materials and Methods

2.1. Bacteria Culture

Bacillus megaterium 2333 and *Lactobacillus casei 76* were provided by the State Institute of Microbe Preservation, DPR Korea. Nutrient broth (NB) and MRS medium were used for enrichment culture of *Bacillus megaterium 2333*, *Lactobacillus casei 76*, respectively. Each media was placed in Erlenmeyer flasks and sterilized at 121 ℃ for 30 min. After cooling to room temperature, each strain was inoculated (NB for Bacillus, MRS for *Lactobacillus*) and incubated 30 ℃ for 36 h at 120 rpm. After enrichment culture, the number of *B. megaterium* and *Lactobacillus* reached 2.6×10^8 and 3.2×10^9 colony forming units (CFU) per milliliter, respectively. The numbers were confirmed by plating serial dilution methods.

2.2. Evaluation of Probiotic Characteristics

2.2.1. The Acid Resistance

The acid resistance of bacterial strain was tested according to the previous method with some modifications [8]. After cultured the bacterial strains on each media for at 35 ℃ for 24 h, bacteria were harvested by centrifuge (10, 000 rpm, 5 min), washed and resuspended in PBS solution at different pH (pH 2.0, pH 2.5, pH 3.0). This solution was incubated at 37 ℃ for 2 h, mimicking intestinal environment. Acid resistance was measured by viable colony counts on each medium.

2.2.2. BILE Salt Resistance

The bile salt resistance of bacterial strain was tested according to the previous method with some modifications [9]. After cultured the bacterial strains on each media for at 35 ℃ for 24 h, bacteria were harvested by centrifuge (10, 000 rpm, 5 min), washed and resuspended in PBS solution (pH 8.0) with 1% bile salts. After incubating at 37 ℃ for 4 h, bile salt resistance was measured by viable colony counts on each medium.

2.2.3. Evaluation of Adhesion Ability to Intestinal Piglet Mucus

Adhesion ability of bacterial strain was tested as previously described method with some modifications [10]. Briefly, the intestinal mucus was collected from the small intestine of healthy piglets. After centrifugation at 10, 000 rpm for 10 min, ethanol was added to the supernatant to precipitate the intestinal mucus polysaccharides. The isolated muco-polysaccharides were diluted to the concentration of 0.5 mg/mL in PBS buffer (pH=7.4). An aliquot (200 μL) of this solution was incubated in polystyrene plate for 12 h to simulate the intestinal mucosal environment. After gently washing with HCl (0.1 M), 200 μL of bacterial dilution solution (about 10^7 CFU/mL) was added and incubated at 39 ℃ for 2 h before washing. Then, viable bacteria numbers were counted by plate serial dilution method from muco-polysaccharide of polystyrene plate. Adhesion was calculated as the number of bacteria recovered after adhesion relative to the number of the bacterial suspension added to the immobilized mucus.

2.2.4. Coexistence Test

Coexistence of *B. megaterium* and *Lactobacillus* was tested by previous method [9]. Two strains were streaked perpendicularly and incubated for 24 h at 37 ℃ in order to evaluate their antagonism.

2.3. Probiotic Bacteria Culture and Supplemental Feed Preparation

After enrichment culture of each strain, supplemental feed was manufactured based on our practical experience. Briefly, corn flour, soybean meal and rice bran were placed in a 6: 3: 1 ratio and sterilized at 121 ℃ for 30 min. Then, this mixture was divided into 4 parts: non-probiotic diet, basal diet with 1% inoculation of *B. megaterium* culture media, basal diet with 1% inoculation of *Lactobacillus* culture media, a combination of 1% *B. megaterium* and 1% *Lactobacillus* culture media and. After inoculation, substrate was covered with vinyl chloride sheet. It was placed in a growth chamber at 32 ℃ for 48 h. After that, 0.1% benzoic acid and 0.3% salt were added, air-dried at temperatures below 40 ℃ and used as supplement feed after grinding. Vitamins, minerals, phytase, whey protein were added to the feed. The non-probiotic supplemental feed was prepared by exactly same method with the exception of inoculation process.

2.4. Chemical Analysis of Supplemental Feed

For the chemical analysis of supplemental feed, a certain amount of supplemental feed was randomly chosen and analyzed according to the previous method [1]. First, samples were dried in an oven at 105 ℃ for 2 h. Crude protein content was measured by the Kjeldahl method. The gross energy content was determined by total combustion in a calorimeter (Parr 1281; Parr Instrument Corp., Moline, IL, USA). Calcium and total phosphorus were calculated according to [11].

2.5. Experiment Animal and Design

Thirty-six suckling piglets (Large White×Duroc, 25 days) were provided from Unjong pig test farm (DPR Korea). These pigs were randomly divided into 4 groups (Group 1, 2, 3, 4) balanced for sex and body weight. They were transferred to pen. Each pen was equipped with a feeder, a faucet and ventilation hole. Group 1 was control (non-probiotic feed), Group 2 was fed a basal diet with *B. megaterium*, Group 3 was provided for *Lactobacillus casei* and Group 4 was for a combination of *B. megaterium* and *Lactobacillus*. The piglets had ad libitum access to feed and water. Average daily feed intake (ADFI) (g/day), Average daily gain (g/day) and Feed conversion ratio (FCR) were measured to compare growth performance of piglets after 4 weeks of experiment. These piglets were weaned at the day 40.

2.6. The Bacterial Flora in Fecal Samples and Incidence of Diarrhea

Incidence of diarrhea was measured according to the previous method [4]. Fecal samples were collected after 3 weeks of experiment. After dilution samples by sterile saline, the numbers of E. coli and *Salmonellae* in fecal samples were enumerated by selective culture method. *E. coli* population was estimated in MacConkey agar, *Salmonellae* were calculated using Salmonella-Shigella agar [1, 9].

2.7. Statistical Analysis

After confirmation of normality (Shapiro-Wilk test), Student's t test and Tukey's HSD Test with a confidence level of 95% were applied. All data were expressed as mean ± SD (standard deviation) and processed with R for windows version.

3. Result and Discussion

3.1. Probiotic Characteristics of B. Megaterium 2333 and Lactobacillus Casei 76

The bacteria should survive in gastric environment in order to function as probiotics. Therefore, acid and bile salt tolerance are important characteristics of successful probiotic bacteria. In this study, survival rate of both bacteria increased with the increase of pH. Although survival rate of *B. megaterium* was higher than that of *Lactobacillus*, both strain showed relatively higher survival rate, demonstrating their good survival ability in gastric environment. Bile salt is known to be toxic to several microorganisms. In bile salt tolerance test, two strains also showed high bile salt tolerance (23.4±3.2%, 19.4±2.6%, respectively), implying their endurance to digestive stress. Adhesion ability to intestinal mucosa is prerequisite requirement for probiotic bacteria. In this experiment, *B. megaterium* exerted relatively lower adhesion rate to mucus. However, two strains had adhesion ability to the mucus, indicating these two bacteria could be used as potential probiotics. *B. megaterium* showed higher survival rate than *Lactobacillus*. This could be attributed to its spore-forming ability, which enables this bacterium endure harsh environment. Evaluation of antagonism of probiotic candidates against each other is of importance to apply them as a combination. In present study, little antagonism was observed on the MRS agar after incubation, indicating these two strains could be combined as a probiotic mixture.

Table 1. *Probiotic characteristics of two strains.*

Probiotic candidates	Survival in acid condition (%)			Bile salt resistance	Adhesion to mucus (%)	Coexistence (inhibition)
	pH 2.0	pH 2.5	pH 3.0			
Bacillus megaterium*2333*	87.4±4.2	90.7±4.4	96.5±5.1	23.4±3.2	34.1±1.1	none
Lactobacillus casei76	81.5±2.2	84.3±2.4	89.4±2.1	19.4±2.6	57.9±2.4	

3.2. Chemical Composition of the Supplemental Feed

Chemical analysis results of supplemental feed were displayed in Table 2.

Table 2. *Chemical composition of supplemental feed.*

Test group	Metabolized Energy (MJ/kg)	Crude protein (%)	Lysine (g/kg)	Methionine (g/kg)	Calcium (g/kg)	Total phosphorus (g/kg)
Group 1 (Control)	13.1±2.0	28.5±2.4	11.4±7.1	8.1±1.7	0.83±0.14	0.71±0.18
Group 2 (B. megaterium)	14.2±3.2	28.9±3.7	12.5±2.2	6.5±1.3	0.78±0.17	0.69±0.19
Group 3 (Lacto-bacillus casei)	14.6±2.2	27.5±2.5	13.7±1.4	7.4±1.2	0.85±0.35	0.72±0.20
Group 4 (Com-bination)	15.1±1.4	26.3±2.7	15.4±3.4	9.5±1.1	0.89±0.16	0.65±0.15

As shown in Table 2, no remarkable differences were observed in different groups in terms of feed composition. From this result, it may be induced that addition of probiotic bacteria to feed did not affect the nutrition value.

Growth performance of piglets

Table 3. *Growth performance of piglets.*

Test Group	Initial weight (kg)	Average daily feed intake (g/day)	Average daily gain (g/day)	Feed conversion ratio (feed/gain)
Group 1 (Control)	4.7±0.2	754±4	0.34±0.13	2.16±0.32
Group 2 (B. megaterium)	4.6±0.3	699±5	0.41±0.14	1.67±0.17
Group 3 (Lactobacillus casei)	4.7±0.2	701±4	0.42±0.17	1.65±0.21
Group 4 (Combination)	4.8±0.1	678±3	0.44±0.20	1.51±0.14

As shown in Table 2, all probiotic groups showed better performance than the control group. No remarkable differences were observed in terms of ADFI and average daily gain between *B. megaterium* and *Lactobacillus* group. However, lowest FCR was measured in a combination group, indicating the same weight gain could be achieved using less feed. In previous studies [12-15], the researchers utilized *Bacillus sp* and *Lactobacillus casei* as a probiotic for piglets and found that these bacteria could enhance average daily weight gain. This was consistent to our research result. This result sums up that application of these two bacterial strains could affect positively on the growth performance of piglets.

Table 4. *Number of pathogens in fecal sample and incidence of diarrhea.*

Test Group	*E.Coli*	Salmonella	Incidence of Diarrhea
Group 1 (Control)	5.82 ±0.04	3.98 ±0.14	11.10 ±0.28
Group 2 (B. megaterium)	2.18 ±0.08	2.41 ±0.26	3.61 ±0.17
Group 3 (Lactobacillus casei)	2.17 ±0.12	2.74 ±0.16	2.72 ±0.29
Group 4 (Combination)	1.01 ±0.04	1.71 ±0.32	1.44 ±0.15

Note: Data were expressed as mean ± SD and log (CFU)

Diarrhea in piglets is the one of the main reasons for economic losses. In this study, probiotic group showed lower diarrhea incidence, demonstrating the benefit of probiotic application. A large number of research were reported to illustrate that probiotic bacteria could reduce the incidence of diarrhea in piglets. Probiotics bacteria could control the intestinal microbiota, consequently control the diarrhea.

E. coli is one of the major pathogens causing diarrhea and *Salmonella* is a typical pathogen to piglets. As shown in the table, the *E. coli* and *Salmonella* content in the probiotic treatment was much lower than that of the control, especially in the combination of the two bacteria. As a result, the incidence of diarrhea was also lower in probiotic group compared to the control. Overall, it can be concluded that application of these bacteria could lower fecal *E. coli* and *Salmonella* content.

4. Conclusion

The results of present study indicate that application of *B. megaterium* and *Lactobacillus casei* may affect positively on piglets rearing. Especially, manufacturing supplemental feed by combing these two bacteria could be beneficial to the improvement of growth performance of piglets. This probiotic supplementation seemingly reduced the incidence of diarrhea. Based on present result, probiotic supplementation feed can be manufactured by a simple and low-cost method, which is suitable in practical condition of swine production industry. However, the exact mechanisms of these probiotic bacteria should be investigated in further research.

Abbreviations

NB	Nutrient Broth
CFU	Colony Forming Unit
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
FCR	Feed Conversion Ratio

Author Contributions

Chang-Gon Sin: designed experiment and wrote manuscript.

Su-Chol Rim: designed experiment and wrote manuscript.

Kwang-Il To: supervised and reviewed.

Jong-Chol Son: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Won-Ju Hwang: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Chang-Su Kim: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Hak-Chol Choe: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Hyok-Won Kim: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Chon-Il Kim: isolated bacteria, tested probiotic characteristics, manufactured supplemental feed and performed comparative test.

Conflicts of Interest

The authors declare no conflicts of interests.

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