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Probiotic properties of genetically engineered *Lactobacillus plantarum* producing porcine lactoferrin used as feed additive for piglets

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ABSTRACT

In this study, a genetically engineered *Lactobacillus* (L.) *plantarum* producing porcine lactoferrin (pLF) was developed and used as feed additive in the daily diet of weaned piglets were investigated, involving in growth performance, intestinal morphology and immunological indices. Results showed that the addition of *L. plantarum*/pPG-pLF in basal diet of weaned piglets significantly increased the average daily gain and feed intake, improved feed efficiency, and reduced incidence of diarrhea, compared to control group received basal diet only. The villus height in duodenum, ileum and jejunum, and the ratio of villus height to crypt depth of piglets fed with *L. plantarum*/pPG-pLF showed significantly increased levels. The number of *Escherichia coli* was decreased and the viable counts of *Bifidobacterium* and *Lactobacillus* were increased in intestine of piglets received *L. plantarum*/pPG-pLF than basal diet only or supplemented with Aureomycin. Moreover, immunological indices were determined that were induced by *L. plantarum*/pPG-pLF and results showed that the levels of total IgG, interleukin-2 and secretory immunoglobulin A were higher in experimented piglets than control group, while the level of interleukin-4 was reduced. The genetically engineered *L. plantarum* developed in this study provided a healthy and eco-friendly agent for weaned piglets against weaning stress and infections.

1. Introduction

Weaning stress in piglets is a serious issue in the swine industry, followed with the increased risk of diseases, small intestinal villus atrophy, intestinal microecological imbalance and mortality [9,11,12]. Moreover, antibiotics as feed additive in the swine industry were widely used to control the incidence of diarrhea and promote growth [6,9], while the long-term abuse of antibiotics have caused the serious antibiotic resistance problems [20]. Therefore, the development of a healthy and eco-friendly agent for the weaned piglets against weaning stress and diseases is of great importance.

Lactoferrin is a natural iron-binding glycoprotein with many physiological functions such as bacteriostatic and antiviral activity [16,35], adjusting the iron balance, promoting the transfer and absorption of iron [18], and regulating immune functions [26,32]. The use of lactoferrin in animals via oral administration has been reported [1,29].

Lactobacillus (L.) *plantarum* is one of the probiotic lactobacilli and is well known for beneficial effects on the health of human and animal, such as antibacterial and immunostimulatory activities [17,21]. *L. plantarum* can survive transit through the upper gastrointestinal tract, and colonize in the intestinal tract and maintain intestinal microecological balance [8]. Moreover, *L. plantarum* has been shown to be a viable mucosal vaccine vector with intrinsic adjuvant activity for the development of oral vaccines [19,37]. We have previously generated porcine lactoferrin (pLF) expressing *L. plantarum* (*L. plantarum*/pPG-pLF) using a recombinant approach. When Compared of improved effect of antibacterial and antiviral activity of four probiotic *Lactobacillus* expressing porcine lactoferrin in mice, and found that *L. plantarum* treatment expressing porcine lactoferrin showed a better beneficial effect on the health of animal Yu et al., 2015. The objective of the current study was to investigate the feasibility of using *L. plantarum*/pPG-pLF as feed additive for the weaned piglets.

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Table 1
Ingredient and nutrient levels of basal diet.

Ingredient	Content (%)
Corn	59.20
Soybean meal	24.00
Fish meal	3.00
Wheat middlings	3.00
$CaHPO_4$	2.00
Limestone	1.00
Whey powder	3.00
NaCl	0.30
Emulsified fatty powder	3.00
Choline chloride	0.10
Lysine	0.34
Methionine	0.06
Premix[a]	1.00
Total	100.00
Nutrient levels	
DE/(MJ/kg)	14.46
CP, %	19.50
Lys, %	1.35
Ca, %	0.80
P, %	0.66

[a] The premix provided the followings in per kg of diet: 2200 IU of vitamin A, 200 IU of vitamin D_3, 16 mg of vitamin E, 1 mg of vitamin K, 200 mg of choline, 6 mg of pantothenic acid, 2 mg of vitamin B2, 0.3 mg of folic acid, 25 mg of nicotinic acid, 6 mg of vitamin B11, 6 mg of vitamin B6, 0.08 mg of biotin, 0.01 mg of vitamin B12, 6 mg of Cu as copper sulfate, 100 mg of Fe as ferrous sulfate, 100 mg of Zn as zinc sulfate, 20 mg of Mn, 0.14 mg of I and 0.3 mg of Se.

2. Materials and methods

The animal protocol for this research was reviewed and approved by the Animal Care and Use Committee of Northeast Agricultural University.

2.1. *Recombinant* L. plantarum *production and identification*

Recombinant *L. plantarum* expressing porcine lactoferrin was generated and cultured as the method previously described Yu et al., 2015, generating the recombinant *L. plantarum*/pPG-pLF. For the expression and identification of the pLF, the *L. plantarum* transformant was cultured in de Man Rogosa and Sharpe (MRS) medium until $OD_{600} \approx 1.0$, and was then induced by xylose for 36 h. The total proteins of the cell pellets and the supernatant of recombinant *L. plantarum* cultures were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Subsequently, the proteins were electrotransferred to a PVDF membrane [4], followed by the incubation with the rabbit anti-pLF antibody diluted at 1:500 (Northeast Agricultural University, Harbin, China) and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody diluted at 1:2000 (Qcbio Science and Technologies, Shanghai, China), respectively. Moreover, the pLF protein secreted into the culture supernatant was quantitatively analyzed with the sera against porcine lactoferrin as the primary antibody using enzyme-linked immunosorbent assay (ELISA) method previously described [5].

2.2. *Animal experiments*

Weaned piglets ($n = 90$; Large White × Landrace; 20-day old) selected from a pig farm (Harbin, China) were divided into three groups and kept in a temperature-controlled nursery room ($24 \pm 1\,^{\circ}C$), respectively. One experimental group of 30 piglets (10 piglets per pen) was fed with basal diet (Table 1) supplemented with 3×10^9 CFU of the transformed *L. plantarum* producing the pLF used as the pLF treatment. Another experimental group of 30 piglets (10 piglets per pen) was fed with basal diet containing 150 mg/kg of Aureomycin used as antibiotic treatment. The group of 30 piglets (10 piglets per pen) received the basal diet only was used as control treatment. The recombinant *L. plantarum*/pPG-pLF and Aureomycin were used as feed additives and the diets were formulated as suggested by the [22] for 5–10 kg piglets and met or exceeded the requirements. The piglets had free access to feed and water throughout the 5-week experiment.

2.3. *Growth performance*

All piglets were individually weighed per week and the feed consumption per pen per day was measured throughout the 5-week experiment. Consequently, the average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (G/F) were determined to evaluate the growth performance of each group. Moreover, the incidence of diarrhea was calculated according to the following formula, where incidence (%) = the number of piglets with diarrhea/(the number of experimental piglets × total experimental days) × 100 [14,27]. The occurrence of diarrhea was defined as definitely unformed, moderately fluid feces or very watery and frothy diarrhea for two consecutive days.

2.4. *Samples collection*

On days 14 after the experiment beginning, blood sample of each piglet was collected in a vacuum tube from each group and placed overnight at 4 °C, after which these blood samples were centrifuged at 2000*g* for 15 min and the sera were stored at −70 °C until required. Subsequently, intestinal tissues of five piglets randomly selected from each treatment group were obtained by laparotomy under general anesthesia via intraperitoneal injection with sodium pentothal solution. The intestinal mucosa scrapings and lavage fluids were collected, and the ileum, duodenum and jejunum were excised, washed with physiological saline and fixed in 10% formaldehyde for histomorphological analysis.

2.5. *Intestinal morphology*

Intestine tissue samples fixed with 10% formaldehyde were prepared using paraffin embedding techniques. And then, histological examination was performed using an Olympus CK 40 microscope (Olympus, Shenzhen, China) as the method previously described [31]. Briefly, the villus height (VH) was measured from the tip of the villus to the villus-crypt junction and the crypt depth (CD) was defined as the depth of invaginations between adjacent villi.

2.6. *Intestinal microbial analysis*

Intestinal mucosal scrapings (approximate 10 g) of the piglets obtained from each treatment group was homogenized in 90 mL of anaerobic dilution solution (ADS) under CO_2 [3], respectively. Following serial 10-fold dilutions in PBS buffer, pH7.4, *Escherichia coli* populations were counted using MacConkey agar plate (Beijing Land Bridge Technology Co., Ltd., Beijing, China), and *Bifidobacterium* populations were counted using *Bifidobacterium* BS Medium (Beijing Shuangxuan Microbe Culture Medium Products Factory, Beijing, China), and *Lactobacillus* was counted using MRS agar plate (Qingdao Hope Bio-Technology CO., LTD., Beijing, China). All count plates were incubated at 37 °C for 24–48 h.

2.7. *Determination of cytokine level*

The levels of lymphokines including interleukin-1 (IL-1), interleukin-2 (IL-2) and interleukin-4 (IL-4) in sera samples, and IgG in sera samples and secretory immunoglobulin A (sIgA) in intestinal mucus were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Meilian Biological