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Effects of *Mycobacterium phlei* on intestinal microecology and serum immunological parameters of weaned piglets

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Abstract: The aim of this study was to determine the effects of *Mycobacterium phlei* in diets (Experiment 1) and investigate the mode of action on the intestinal microecology of weaned piglets (Experiments 1 and 2). In Experiment 1, a total of 96 commercial cross-bred pigs [(Duroc × Yorkshire) × Landrace], weaned at 21 days, were assigned to two treatments in a completely randomized design with a single factorial arrangement of 40 days of supplementation (nonsupplemented vs. supplemented with 0.4% heat-killed *M. phlei*). The results showed that *M. phlei* has the capability to improve the intestinal microecology of weaned piglets. The serum concentrations of IL-2, IL-12, and TNF-α from the experimental group were significantly higher than in the control group ($P < 0.01$). In Experiment 2, the antibacterial effect of 5 different extracts (pure water, 95% ethanol, chloroform, ethyl acetate, and n-butanol) from *M. phlei* was assayed by the Plat stiletto method. The results showed that these extracts had hardly any antibacterial effect on *E. coli.* Our results indicate that *M. phlei* can improve the intestinal microecology of weaned piglets not because of its antibacterial effects, but because of these immune factors stimulated by the microecology of weaned piglets.

Key words: *Mycobacterium phlei*, intestinal microecology, antibacterial effect

1. Introduction

Weaning is associated with a major shift in the gut microbiota of piglets in response to dietary interventions (1). To assist in overcoming the postweaning growth check, antibiotics and/or mineral compounds, such as ZnO, are traditionally added to feed for weanling piglets. However, due to the possible contribution of in-feed antibiotics to the development of antibiotic-resistant strains of bacteria, the European Union implemented a full ban on in-feed antibiotics usage in livestock diets in January 2006. There is also pressure in other pig-producing regions of the world to minimize or completely eliminate the inclusion of in-feed antibiotics in livestock diets, for a number of different reasons (2). Hence, natural alternatives are gradually gaining widespread use. The application of microbial agents in natural medicine has become a research hotspot because of the low cost, rapid large-scale production, and ease of technological transformation.

Mycobacterium phlei, which was first identified in 1889, is a fast-growing saprophyte that is widely distributed in nature and nonpathogenic to both humans and animals (3). Several experiments have confirmed that the cell wall (4), DNA (5), unmethylated CPG (6), and polysaccharides (7) of *M. phlei* have antitumor and antiradiation activities. Therefore, *M. phlei* is widely used for treatment of superficial bladder cancer and recurrent respiratory tract infections (8,9). In a study by Bera et al. (10), heat-killed *M. phlei* fed to broiler chickens infected with *Eimeria tenella* at a dose of 10 mg per animal on alternate days for a total of 4 doses resulted in a higher body weight gain as compared with healthy controls or a coccidia-infected group. Immunoglobulin A concentrations in serum and bile were also higher in the *M. phlei*-supplemented group at 7 days post challenge with coccidian oocysts when compared with coccidia-infected and healthy control groups (10). These series of experiments indicated that *M. phlei* has the potential to reduce effects of harmful disease-causing microorganisms and maintain homeostatic function in vivo.

Based on the findings of these cited studies, we hypothesized that *M. phlei* could be used as a dietary supplement for weaned piglets to modulate the intestinal microflora. Therefore, the aims of this study were to observe the modulating effect of *M. phlei* on the

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intestinal microecology of piglets and then to elucidate possible mechanisms through the serum immunological parameters of weaned piglets and antibacterial effects of *M. phlei*.

2. Materials and methods

2.1. Experiment 1: Effects of *M. phlei* on intestinal microflora and serum immunological parameters of piglets

2.1.1. Animals and treatment

The study protocol was approved by the Animal Care and Use Committee of Hunan Agricultural University (HAU201408). Commercial cross-bred pigs [n = 96; (Duroc × Yorkshire) × Landrace], weaned at 21 days, were randomly allocated to two treatment groups (6 replicate pens per treatment and 8 piglets per pen): a control group (basal diet) and an experimental group (basal diet + 0.4% *M. phlei*). The basal diet met National Research Council (11) nutrient requirements for swine (Table 1). *M. phlei* was inactive in the form of vacuumed and freeze-dried powder, supplied free of charge by the Hunan Sheng Yakai Technology Company (Changsha, China).

2.1.2. Sample collection and analysis

The piglets were fed the experimental diets for 40 days and then weighed, and feed consumption was measured. Daily feed intake, daily body weight gain, and feed conversion efficiency were calculated. At the end of the trial period, blood samples from four piglets from each pen were collected from the left jugular vein, and plasma samples, obtained after centrifugation, were stored at –20 °C. An enzyme-linked immunosorbent assay was used to measure concentrations of interleukin (IL)-2, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. These four animals were then sacrificed to sterilely collect intestinal fecal and chyme samples for the analysis of *Escherichia coli* and *Lactobacillus* content. One gram of sample was diluted with 9 mL of 1% peptone solution and homogenized. Counts of viable bacteria in feces and chyme samples were determined by plating serial tenfold dilutions (in 1% peptone solution) onto Lactobacillus Medium III agar plates for lactic acid bacteria and MacConkey agar plates for *Enterobacteriaceae* (12).

2.1.3. Statistical analyses

Data were analyzed by one-way analysis of variance using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

2.2. Experiment 2: Antibacterial effect of *M. phlei*

2.2.1. Preparation of crude extracts

Five samples of *M. phlei* bacterial powder (3 g each) were mixed with 30 mL of pure water, 95% ethanol, chloroform, ethyl acetate, and n-butanol, respectively, and then oscillated at 140 rpm for 2 h at 40 °C in an oscillating water bath (Guohua Co., Ltd., Changzhou, China). The mixture was filtered and the filtrate was collected into 5 tubes. The remaining filter residue was suspended in 20 mL of the same liquid used previously and then oscillated

Table 1. The composition and nutrient levels of the basal diet (air-dried basis, %).

Ingredients	Proportion	Nutrient levels	
Corn	66.5	DE (MJ/Kg)	13.50
Soybean meal	19.0	CP (%)	18.00
Soybean oil	3.0	Ca (%)	0.72
Glucose	2.5	TP (%)	0.60
Fish meal	2.0	Salt (%)	0.50
Puffed soybean	4.0	Lysine (%)	1.20
Limestone	0.5	Methionine (%)	0.40
Calcium lactate	0.5		
Chlorination choline	0.1		
Lysine	0.3		
Methionine	0.1		
Salt	0.3		
Zinc oxide	0.3		
$NaHCO_3$	0.3		
$Ca(H_2PO_4)_2$	0.6		
Total	100		

and filtered. The filtrate was collected and mixed with the filtrate collected previously. The filtrate mixture was evaporated under reduced pressure in a rotary evaporator (Rotavapor R-114; BÜCHI Labortechnik AG, Flawil, Switzerland) at 45 °C and aliquoted into 5 portions of completely dry extracts. The extracts were then dissolved in 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) before use (13).

2.2.2. Tested bacterial strains

E. coli was obtained from Hunan Agriculture University, Changsha, China. The bacteria were maintained on beef extract peptone medium slants (0.3% beef extract, 1% peptone, 0.5% NaCl, 2% agar, pH 7.0–7.2) at 4 °C. Before the experimentation, the bacteria were grown on Müller-Hinton agar (MH; Merck KGaA, Darmstadt, Germany) at 37 °C for 18–24 h.

2.2.3. Determination of antimicrobial activity

Five well-isolated *E. coli* colonies were suspended in 250 mL of brain-heart infusion broth (Difco Laboratories, Inc., Detroit, MI, USA) and incubated at 37 °C for 6 h until entering the logarithmic growth phase and then subcultured on MH agar overnight. The inoculum was finally adjusted to 10^8 CFU mL^{-1} using the McFarland standard (14). Fresh cultures of the microorganisms (100 µL) were inoculated on MH agar. Bacteriostatic activity of the 5 crude extracts was assayed by the Plat stiletto method and the steps were as follows: 5 holes (diameter, 6 mm) per culture dish were evenly placed and 40 µL of the 5 crude extracts, containing 4 mg of crude extract, was applied drop-wise through the 5 holes. The plates were then incubated at 37 °C for 24 h and the diameters of inhibition zones were measured. Each assay was repeated 3 times and the positive and negative tests were performed under the same conditions (15).

3. Results

3.1. Effects of *M. phlei* on growth performance of weaning piglets

A shown in Table 2, the growth performances of weaning piglets in the control and experimental groups were quite similar. Notably, the incidence of diarrhea among piglets in the experimental group was lower than that of the control group, although not significantly.

3.2. Effects of *M. phlei* on intestinal microflora

As shown in the Figure, the number of *E. coli* in fecal samples from piglets in the experimental group was remarkably lower than from those in the control group ($P < 0.05$). The *E. coli* content in the cecum of piglets in the control group was 3.5-fold greater than that of the experimental group and 6.12-fold greater than in the colon. The *Lactobacillus* content in the control group was remarkably lower than that of the experimental group ($P < 0.05$).

3.3. Effects of *M. phlei* on serum immunological parameters

As shown in Table 3, there were differences in serum immunological parameters between the control and experimental groups, especially IL-2, IL-12, and TNF-α concentrations, which were significantly or extremely significantly higher in the experimental group.

3.4. Antimicrobial activity of *M. phlei*

As shown in Table 4, the ethanol, ethyl acetate, butanol, and chloroform extracts of *M. phlei* had barely any inhibitory effects.

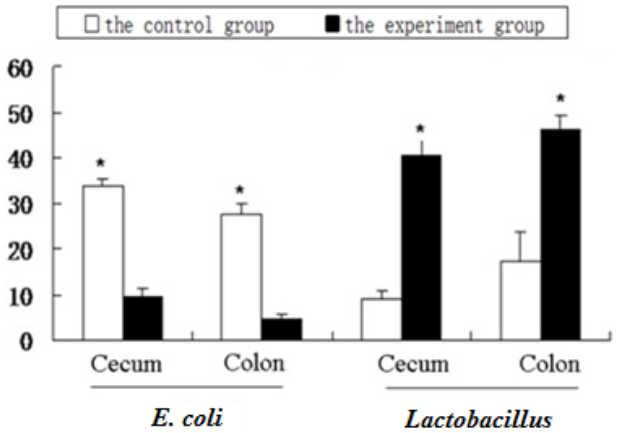

Figure. Effect of *M. phlei* on the intestinal microflora of weaning piglets.

Table 2. Effects of *M. phlei* on the growth performance of weaning piglets.

Items	Control group	Experiment group
Initial weight (kg)	8.65 ± 0.34	8.64 ± 0.29
Final weight (kg)	21.98 ± 0.78	22.69 ± 0.53
Average daily gain (kg/day)	0.44 ± 0.01	0.47 ± 0.01
Average daily feed intake (kg/day)	0.78 ± 0.01	0.80 ± 0.01
The ratio of feed and meat	1.75 ± 0.02	1.71 ± 0.02
Diarrhea rate (%)	0.83 ± 0.53	0.42 ± 0.19

Table 3. Effects of *M. phlei* on the serum immunological parameter of weaning piglets.

Items	Control group	Experiment group
IL-2 (pg/mL)	20.17 ± 3.74^{b}	37.08 ± 7.20^{a}
IL-12 (pg/mL)	0.72 ± 0.21^{B}	9.70 ± 1.61^{A}
TNF-α (ng/L)	14.38 ± 1.19^{b}	21.15 ± 3.00^{a}
IFN-γ (pg/mL)	75.10 ± 5.78	87.97 ± 5.51

Values in a row with different superscripts are significantly different.

Table 4. Inhibition zone of different extracts to *Escherichia coli* (antibacterial circle diameter d = mm).

Bacteriostatics	*Escherichia coli*
Aqueous extracts	6.0 ± 0.1
95% ethanol extracts	8.0 ± 0.2
Chloroform extracts	6.0 ± 0.1
Ethyl acetate extracts	12.0 ± 0.2
Butanol extracts	10.0 ± 0.3
Dimethyl sulfoxide	6.0 ± 0.1
Amikacin	15.0 ± 0.3

4. Discussion

Although *M. phlei* is known to convey antitumor and antiradiation effects, few studies have investigated the capability of this bacterium to modulate intestinal microflora. Our results showed that *M. phlei* could improve the intestinal microecological health of weaned pigs and provide strong protection against some pathogenic microorganisms. The incidence of diarrhea among piglets in the experimental group was lower than that of the control group (0.42 ± 0.19% vs. 0.83 ± 0.53%, respectively). The effect of a water-soluble fraction of *M. phlei* can steadily decrease the total bacterial count in bovine subclinical mastitis (16). Immunopotentiation with *M. phlei* was shown to significantly reduce diarrhea-associated morbidity and mortality of weaned piglets (17). This result is also consistent with a report by Loh et al. (18). Our results indicated that *M. phlei* may inhibit the growth of harmful microbes in weanling pigs. In order to clarify the antibacterial effects of *M. phlei*, bacteriostatic tests were carried out with extracts dissolved in different polar solvents, but none of the above extracts had obvious bacteriostatic effects.

We also identified factors that modulate intestinal microflora and found that serum immunomodulatory molecules induced changes in the intestinal microflora of weaned piglets following stimulation with *M. phlei*. When *M. phlei*, an immunomodulatory molecule, was absorbed, the concentrations of serum IL-2, IL-6, IL-12, TNF-α, and IFN-γ gradually changed. Filion et al. (19) reported that the cell wall contains the majority of immunoactive constituents, which stimulate monocytes to secrete TNF-α through the nuclear factor kappa B signaling pathways and monophasic action potentials. Oligonucleotide DNA on the cell surface could act to produce excess IL-12 (20). IL-12 is a heterodimeric immunoregulatory cytokine that promotes proliferation of T cells and natural killer cells. The *M. phlei* cell wall complex directly induces apoptosis of human bladder cancer cells by producing IL-12 (19). In addition, IL-12 upregulates production of other cytokines, including IFN-γ and TNF-α (21). According to a report by Bera et al. (10), heat-killed *M. phlei* plays a beneficial role as an immunostimulant against cecal coccidiosis in broiler chickens. Inhalation of *M. phlei* can also reduce airway inflammation in asthmatic mice (22). Moreover, a large number of clinical trials showed that *M. phlei* can be used in the treatment of disease of the respiratory and urogenital tracts, such as tuberculosis, bladder cancer, and condyloma acuminatum. Our results indicated that the

immunomodulatory molecules may be the main factors that induce changes in the intestinal microflora of weaned piglets.

In conclusion, *M. phlei* can improve the intestinal microecology of weaned piglets by promoting stimulation of secreted immune factors, rather than through antibacterial effects.

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