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Effects of Pseudostellaria heterophylla Stem and Leaf Polysaccharides on Intestinal Immune Function, Intestinal Mucosal Morphology and Structure, and Cecal Content Microbiota in Weaned Piglets: Postprint

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Abstract

This experiment aimed to investigate the effects of dietary supplementation with Pseudostellaria heterophylla stem and leaf polysaccharides on intestinal immune function, intestinal mucosal morphology, and cecal content microbiota in weaned piglets. A total of 120 25-day-old weaned "Large White × Landrace" crossbred piglets were selected and randomly divided into 4 groups according to similar body weight, with 3 replicates per group and 10 piglets per replicate. The control group was fed a basal diet, while the experimental groups were supplemented with 500 (0.05% polysaccharide group), 1,000 (0.10% polysaccharide group), and 1,500 mg/kg (0.15% polysaccharide group) of Pseudostellaria heterophylla stem and leaf polysaccharides on top of the basal diet. The experimental period lasted 30 days. The results showed that: compared with the control group, the content of interleukin-4 (IL-4) in the duodenum was significantly increased in all experimental groups ($P<0.05$), the content of interferon-γ (IFN-γ) in the duodenum was significantly increased in the 0.10% polysaccharide group ($P<0.05$), and the content of secretory immunoglobulin A (SIgA) in the ileum was significantly increased in both the 0.10% and 0.15% polysaccharide groups ($P<0.05$); the villus height in the duodenum and jejunum was significantly or extremely significantly increased in the 0.10% and 0.15% polysaccharide groups ($P<0.05$ or $P<0.01$), and the villus height/crypt depth (V/C) ratio in the duodenum was significantly increased ($P<0.05$); the villus height in the ileum was significantly increased in the 0.15% polysaccharide group ($P<0.05$); the number of Escherichia coli in cecal contents was significantly decreased ($P<0.05$), while the number of Lactobacillus was significantly increased ($P<0.05$) in all experimental groups. It can be concluded that dietary supplementation with

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Pseudostellaria heterophylla stem and leaf polysaccharides can enhance intestinal immune function, increase small intestinal villus height and V/C ratio, regulate cecal content microbiota structure, and maintain intestinal microecological balance in weaned piglets; the appropriate supplementation level is 0.10%.

Full Text

Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Intestinal Immune Function, Intestinal Mucosal Morphology and Cecum Contents Flora of Weaned Piglets

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Abstract: This study was conducted to investigate the effects of dietary Radix pseudostellariae stem and leaf polysaccharide (RpSLp) on intestinal immune function, intestinal mucosal morphology and cecum contents flora of weaned piglets. One hundred and twenty 25-day-old weaned piglets (Large White × Landrace) with similar body weight were randomly allocated into four groups with three replicates per group and ten piglets per replicate. Piglets in the control group were fed a basal diet, while those in the experimental groups were fed the basal diet supplemented with 500 (0.05% polysaccharide group), 1,000 (0.10% polysaccharide group) and 1,500 mg/kg (0.15% polysaccharide group) of RpSLp, respectively. The experimental period lasted for 30 days. The results showed that, compared with the control group, the duodenal interleukin-4 (IL-4) content in all experimental groups was significantly increased ($P<0.05$), the duodenal interferon-γ (IFN-γ) content in the 0.10% polysaccharide group was significantly increased ($P<0.05$), and the ileal secretory immunoglobulin A (SIgA) content in both 0.10% and 0.15% polysaccharide groups was significantly increased ($P<0.05$). The villus height in duodenum and jejunum of the 0.10% and 0.15% polysaccharide groups was significantly or extremely significantly increased ($P<0.05$ or $P<0.01$), and the villus height to crypt depth (V/C) value in duodenum was also significantly increased ($P<0.05$). The ileal villus height in the 0.15% polysaccharide group was significantly increased ($P<0.05$). The number of Escherichia coli in cecum contents of all experimental groups was significantly decreased ($P<0.05$), while the number of Lactobacillus was significantly increased ($P<0.05$). It can be concluded that dietary RpSLp can improve intestinal immune function, increase small intestinal villus height and

V/C value, regulate cecum contents flora structure, and maintain intestinal microecological balance in weaned piglets. The appropriate supplementation level is 0.10%.

Key words: Radix pseudostellariae stem and leaf polysaccharide; weaned piglets; intestinal immune function; intestinal mucosal morphology; cecum contents flora

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The small intestine serves as a crucial digestive and absorptive organ as well as an immune organ in animals, possessing both the functions of nutrient absorption and defense against pathogen invasion [1]. Stress factors such as weaning, diet change, and pen transfer often cause rapid villus atrophy in piglets, leading to diarrhea and other symptoms [2]. Therefore, maintaining stable intestinal internal environment in weaned piglets is critical in husbandry practices. Studies have found that adding traditional Chinese medicine polysaccharides to diets can alleviate weaning stress, improve immune function and antioxidant capacity, and modulate intestinal microbiota in weaned piglets [3-6]. Radix pseudostellariae, an important economic medicinal herb in Zherong County of Fujian Province, has the efficacy of replenishing qi and generating body fluid, and is commonly used to treat anorexia and fatigue caused by spleen and stomach qi deficiency [7]. Our previous research found that Radix pseudostellariae polysaccharide could antagonize cyclophosphamide-induced intestinal mucosal injury and enhance intestinal immunity in mice [8]. Lei et al. [9] reported that Radix pseudostellariae stems and leaves could reduce the adverse effects of stress on growth and development of weaned piglets, thereby improving anti-stress capacity. Currently, research on Radix pseudostellariae application mainly focuses on its root tuber, while the stems and leaves are discarded. However, studies have shown that the polysaccharide content in Radix pseudostellariae stems and leaves can reach 5%~10% [10], yet the application of RpSLp in weaned piglets is rarely reported. This experiment supplemented RpSLp in the basal diet of weaned piglets and examined changes in intestinal tissue cytokines including interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-γ (IFN-γ), secretory immunoglobulin A (SIgA) content, intestinal morphology, and cecum contents flora to explore its intestinal health effects and potential mechanisms. The results provide scientific basis for the application of RpSLp in weaned piglets and reference for further development of Radix pseudostellariae stems and leaves.

1.1 Experimental Materials

Radix pseudostellariae stem and leaf polysaccharide powder was purchased from Shaanxi Tengmai Biotechnology Co., Ltd. Using glucose as the standard, the polysaccharide content was determined to be 70% by the phenol-sulfuric acid method.

1.2 Main Instruments

The main instruments included an automatic radioimmunoassay counter (XH-6020, Xi' an Nuclear Instrument Factory), an automatic microplate reader (STATFAX-2100, Awareness Technology Inc., USA), a fluorescence quantitative PCR system (C1000, Bio-Rad, USA), an electrophoresis apparatus (DYY-2C, Beijing Liuyi Instrument Factory), a gel imaging system (GL200, Kodak), a rapid mixer (Sk-1, Jintan Kexi Instrument Co., Ltd.), and a digital constant temperature water bath (HH-2, Guohua Electric Appliance Co., Ltd.).

1.3 Experimental Design

One hundred and twenty 25-day-old weaned piglets with similar parity and body weight were selected and randomly divided into four groups with three replicates per group and ten piglets per replicate, following the principle of similar body weight with half male and half female. The control group was fed a basal diet; the 0.05% polysaccharide group was fed the basal diet supplemented with 500 mg/kg RpSLp; the 0.10% polysaccharide group was fed the basal diet supplemented with 1,000 mg/kg RpSLp; and the 0.15% polysaccharide group was fed the basal diet supplemented with 1,500 mg/kg RpSLp. During the experiment, all piglets had free access to dry powder feed and water, and were dewormed and vaccinated according to routine farm management procedures. The pre-trial period was 5 days, and the experimental period lasted for 30 days. The composition and nutrient levels of the basal diet are shown in Table 1 .

1.4 Sample Collection and Processing

On the final day of the experiment, one piglet was randomly selected from each replicate for slaughter. The duodenum, jejunum, and ileum were collected. After rinsing with phosphate buffer solution (PBS), each intestinal segment was divided into two portions: one portion was fixed with 4% paraformaldehyde for intestinal morphological analysis, and the other was placed in self-sealing bags and stored at -80°C for determination of intestinal tissue cytokines (IL-2, IL-4, and IFN-γ) and SIgA content. Meanwhile, cecum contents were aseptically collected, aliquoted into EP tubes, and stored at -80°C for cecum flora analysis.

1.5 Determination of Intestinal Tissue Cytokines and SIgA Content

The contents of IL-2, IL-4, and IFN-γ in different intestinal segments were determined by enzyme-linked immunosorbent assay (ELISA), while SIgA content was measured by radioimmunoassay (RIA). Detailed procedures followed the kit instructions.

1.6 Determination of Intestinal Mucosal Morphology and Intraepithelial Lymphocyte Count

Small intestinal tissues preserved in 4% paraformaldehyde were processed through washing, dehydration, clearing, paraffin infiltration, embedding, sectioning, mounting, drying, and dewaxing, followed by hematoxylin-eosin (HE) staining. Two fields of view were selected from each section, and ten intestinal villi were continuously measured in each field. Image-Pro Plus 6.0 software was used to calculate villus height and crypt depth, and to determine the villus height to crypt depth (V/C) value. Under light microscopy, five typical fields of view (with intact and straight villi) were selected from duodenum, jejunum, and ileum sections to calculate the number of intraepithelial lymphocytes per 100 intestinal epithelial columnar cells.

1.7.1 Total DNA Extraction and Purity Identification from Samples

Total DNA was extracted from cecum contents of weaned piglets in each group using a fecal genomic DNA extraction kit (spin column type). The concentration and purity of extracted DNA were determined by a micro-ultraviolet spectrophotometer. DNA should show a significant absorption peak at OD260, with an OD260/OD280 ratio between 1.7 and 1.9.

1.7.2 Primer Design

Specific primers for total intestinal bacteria, Escherichia coli, and Lactobacillus were designed according to Xu et al. [11]. Primer sequences and fragment sizes are shown in Table 2 . Primers were synthesized by Shanghai Bioengineering Co., Ltd.

1.7.3 Primer Validation

Using extracted total intestinal DNA as template, PCR reactions were performed for primers of total bacteria, E. coli, and Lactobacillus. The total PCR reaction volume was 25 L, containing 12.5 L mix, 8.5 L ddH2O, 2 L template, 1 L forward primer, and 1 L reverse primer. PCR conditions were as follows: pre-denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s for total bacteria and Lactobacillus (60°C for E. coli), and extension at 72°C for 30 s; final extension at 72°C for 10 min. PCR products were subjected to gel electrophoresis and analyzed using a gel imaging system.

1.7.4 Real-Time Fluorescence Quantitative PCR

A 12.5 L reaction system was used: 6.25 L GoTaq® qPCR Master Mix, 0.25 L CRX Reference Dye, 4.5 L Nuclease-Free Water, 0.25 L each of forward and

reverse primers, and 1 L DNA template. The GoTaq® qPCR Master Mix kit was purchased from Promega. Reaction conditions were: pre-denaturation at 98°C for 2 min; 39 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 10 s, and extension at 68°C for 30 s.

1.7.5 Fluorescence Relative Quantification Analysis

Each DNA sample was subjected to fluorescence quantitative PCR to obtain Ct values. The 2^(-ΔΔCt) method was used to calculate and analyze changes in E. coli and Lactobacillus among different groups, where ΔΔCt = [Ct_m(test sample) - Ct_n(test sample)] - [Ct_m(control sample) - Ct_n(control sample)], with m representing the target gene and n representing the reference gene. In this experiment, 16S rDNA of total bacteria served as the reference gene, while 16S rDNA of E. coli and Lactobacillus served as target genes. Changes in E. coli and Lactobacillus numbers in treatment groups were expressed as ratios relative to the control group, with the relative value of PCR products in the control group set as 1. The 2^(-ΔΔCt) method was used to calculate relative quantities for each group.

1.8 Statistical Analysis

Experimental results were expressed as mean ± standard deviation (mean±SD). One-way ANOVA was performed using SPSS 17.0 statistical software, and LSD test was used for multiple comparisons.

2.1 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Duodenal Cytokines and SIgA Content in Weaned Piglets

As shown in Table 3 , compared with the control group, duodenal IL-4 content in the three polysaccharide groups was increased by 17.81% ($P<0.05$), 27.39% ($P<0.05$), and 15.07% ($P<0.05$), respectively, with no significant differences among the three polysaccharide groups ($P>0.05$). The duodenal IFN-γ content in the 0.10% and 0.15% polysaccharide groups was increased by 48.79% ($P<0.01$) and 9.88% ($P>0.05$), respectively, with the 0.10% polysaccharide group being extremely significantly higher than the 0.05% and 0.15% polysaccharide groups ($P<0.01$). No significant differences were observed in duodenal IL-2 and SIgA content among the three polysaccharide groups compared with the control group ($P>0.05$).

2.2 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Jejunal Cytokines and SIgA Content in Weaned Piglets

As shown in Table 4 , compared with the control group, the jejunal IFN-γ content in the 0.10% polysaccharide group was increased by 27.67% ($P<0.05$),

which was significantly higher than that in the 0.05% and 0.15% polysaccharide groups (P<0.05). The jejunal SIgA content in the 0.10% polysaccharide group was increased by 30.31% (P<0.05), also significantly higher than the 0.05% and 0.15% polysaccharide groups (P<0.05). No significant differences were observed in jejunal IL-2 and IL-4 content among the three polysaccharide groups compared with the control group (P>0.05).

2.3 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Ileal Cytokines and SIgA Content in Weaned Piglets

As shown in Table 5 , compared with the control group, ileal IFN-γ content in the three polysaccharide groups was increased by 18.82% (P<0.05), 25.14% (P<0.05), and 18.96% (P<0.05), respectively, with no significant differences among the polysaccharide groups (P>0.05). The ileal SIgA content in the 0.10% and 0.15% polysaccharide groups was increased by 19.81% (P<0.05) and 8.21% (P<0.05), respectively, which were significantly higher than that in the 0.05% polysaccharide group (P<0.05). No significant differences were observed in ileal IL-2 and IL-4 content among the three polysaccharide groups compared with the control group (P>0.05).

2.4 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Duodenal Morphology and Intraepithelial Lymphocyte Count in Weaned Piglets

As shown in Table 6 , compared with the control group, duodenal villus height in the 0.10% and 0.15% polysaccharide groups was increased by 26.36% (P<0.01) and 23.93% (P<0.01), respectively, which were extremely significantly higher than that in the 0.05% polysaccharide group (P<0.01). The V/C values in the three polysaccharide groups were increased by 23.02% (P<0.05), 30.93% (P<0.05), and 30.21% (P<0.05), respectively, with no significant differences among polysaccharide groups (P>0.05). No significant differences were observed in crypt depth and intraepithelial lymphocyte count among the three polysaccharide groups compared with the control group (P>0.05).

2.5 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Jejunal Morphology and Intraepithelial Lymphocyte Count in Weaned Piglets

As shown in Table 7 , compared with the control group, jejunal villus height in the 0.10% and 0.15% polysaccharide groups was increased by 14.08% (P<0.05) and 17.41% (P<0.05), respectively, which were significantly higher than that in the 0.05% polysaccharide group (P<0.05). No significant differences were observed in jejunal crypt depth, V/C value, and intraepithelial lymphocyte

count among the three polysaccharide groups compared with the control group (P>0.05).

2.6 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Ileal Morphology and Intraepithelial Lymphocyte Count in Weaned Piglets

As shown in Table 8 , compared with the control group, ileal villus height in the 0.15% polysaccharide group was increased by 20.18% (P<0.05), which was significantly higher than that in the 0.05% and 0.10% polysaccharide groups (P<0.05). No significant differences were observed in ileal crypt depth, V/C value, and intraepithelial lymphocyte count among the three polysaccharide groups compared with the control group (P>0.05).

2.7 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Cecum Contents Flora in Weaned Piglets

As shown in Figure 1 [Figure 1: see original paper], compared with the control group, all three polysaccharide groups significantly decreased the number of E. coli in cecum contents (P<0.05) and significantly increased the number of Lactobacillus (P<0.05), with the 0.10% polysaccharide group showing the best effect.

3.1 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Small Intestinal Cytokines and SIgA Content in Weaned Piglets

SIgA is the primary effector molecule in the lamina propria of small intestinal mucosa that participates in immune responses. When animals are stimulated by antigens, the mucosal layer secretes large amounts of SIgA, which blocks pathogen attachment to the mucosal surface by mixing with mucus, thereby exerting its mucosal immune function [12]. Intestinal mucosal interleukins are also important components of mucosal immunity. IL-2 can promote proliferation and differentiation of T and B lymphocytes and produce specific antibodies, thereby enhancing SIgA-mediated intestinal mucosal immune function [13]. IFN-γ can exert its mucosal immune function by promoting IL-2 production. Zhang et al. [14] reported that Si Jun Zi Tang total polysaccharide could promote SIgA secretion in immunosuppressed mice; Wang et al. [15] found that Laminaria polysaccharide could increase intestinal mucosal SIgA secretion; Ji et al. [16] demonstrated that Danggui Buxue polysaccharide could promote secretion of IL-2, IL-4, and mucosal SIgA in mice; Zhang et al. [17] showed that Taishan Cordyceps polysaccharide could increase intestinal mucosal SIgA secretion in immunosuppressed mice. The present results indicate that dietary RpSLp can increase intestinal mucosal IL-2, IL-4, IFN-γ, and SIgA contents to varying

degrees in weaned piglets, suggesting that RpSLp can promote proliferation and differentiation of intestinal secretory immune cells and enhance systemic immunity. Comparison among the three intestinal segments revealed that the effect of RpSLp on intestinal mucosal immune function was most significant in the duodenum, possibly because duodenal mucosa contains more immune cells and is more sensitive to polysaccharide stimulation compared with jejunum and ileum.

3.2 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Small Intestinal Morphology and Intraepithelial Lymphocyte Count in Weaned Piglets

The small intestine serves as an important digestive and immune organ in animals, with functions of nutrient absorption and defense against pathogen invasion. Villus height and crypt depth can reflect the number of mature epithelial cells and affect nutrient absorption [18]. The V/C value can comprehensively reflect small intestinal nutrient absorption capacity; an increased ratio indicates enhanced digestive and absorptive function and better growth performance [19]. Intestinal mucosal intraepithelial lymphocytes participate in intestinal immune regulation, and the number of lymphocytes per unit area can reflect the strength of intestinal immune function. Wang et al. [20] reported that jujube polysaccharide could increase intestinal villus height and decrease crypt depth in nursery pigs; Huang et al. [21] found that Astragalus polysaccharide could increase intestinal villus height, decrease crypt depth, and increase intraepithelial lymphocyte count in tilapia; Shi et al. [22] demonstrated that Lycium barbarum polysaccharide could promote integrity of intestinal mucosal barrier and increase intraepithelial lymphocyte count in mice. The present results indicate that dietary RpSLp can extremely significantly increase duodenal villus height, significantly increase duodenal V/C value, and moderately increase intestinal mucosal intraepithelial lymphocyte count in weaned piglets. This suggests that RpSLp has the ability to regulate intestinal morphology, promote nutrient digestion and absorption, and enhance intestinal immune function.

3.3 Effects of Radix pseudostellariae Stem and Leaf Polysaccharide on Cecum Contents Flora in Weaned Piglets

Escherichia coli is an opportunistic pathogen that does not cause disease when the intestinal internal environment is stable. Only when animals are stimulated by external factors that disrupt homeostasis, leading to massive proliferation of E. coli, will symptoms such as diarrhea occur, reducing resistance and growth performance [11]. Lactobacillus, as a beneficial bacterium in the animal intestine, can regulate intestinal flora balance and promote digestion and absorption of nutrients such as proteins. In terms of immune regulation, Lactobacillus also plays an important role, not only increasing superoxide dismutase activity

but also inhibiting cholesterol absorption and reducing blood lipids. Wang et al. [20] reported that dietary supplementation with 1,000 and 1,500 mg/kg jujube polysaccharide could promote proliferation of Lactobacillus and Bifidobacterium and inhibit E. coli reproduction in piglet intestines; Feng et al. [23] found that purslane polysaccharide could increase Lactobacillus and Bifidobacterium numbers in ulcerative colitis mice; Zhang et al. [17] demonstrated that Taishan Cordyceps polysaccharide could increase Lactobacillus and Bifidobacterium numbers and decrease E. coli and Enterococcus numbers in immunosuppressed mice. The present results show that dietary RpSLp can decrease cecal E. coli numbers and increase Lactobacillus numbers in weaned piglets. However, as shown in Figure 1 [Figure 1: see original paper], higher supplementation levels are not necessarily better, with 0.10% being the most appropriate level. This may be because at this supplementation level, RpSLp can better regulate cecal pH, maintaining an acidic intestinal environment that promotes proliferation of beneficial bacteria such as Lactobacillus while inhibiting E. coli proliferation to some extent.

Dietary supplementation with Radix pseudostellariae stem and leaf polysaccharide can improve intestinal immune function, increase small intestinal absorption area, modulate intestinal flora structure, help weaned piglets smoothly transition through weaning stress, and promote growth and development. The appropriate supplementation level is 0.10%.

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