Effects of Lactobacillus plantarum-Taishan Pine Pollen Polysaccharide Microcapsules on Growth and Intestinal Environment in Mice: A Postprint

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Abstract

Lactobacillus plantarum, as a microecological feed additive, is susceptible to adverse factors such as gastric acid, bile acids, and digestive enzymes in the digestive tract after administration. Taishan pine pollen polysaccharide (TPPPS), as a water-soluble plant polysaccharide, is also vulnerable to degradation by gastric acid and enzymes. To protect the biological activity of Lactobacillus plantarum and TPPPS, and to explore whether a synergistic effect exists between them, this study prepared microcapsules using Lactobacillus plantarum and TPPPS as core materials, optimized the encapsulation process through orthogonal experiments, and evaluated the properties of microcapsules prepared under optimal conditions. Forty-eight specific pathogen free (SPF) mice weighing approximately 20 g were selected and randomly divided into 4 groups, with 12 mice per group. The four groups of mice were orally administered 1 mL of Lactobacillus plantarum, Lactobacillus plantarum + 0.5% TPPPS, microcapsules, or phosphate buffer saline (PBS) daily. The feeding trial lasted for 42 days. Every week, 3 mice were randomly selected from each group for weighing and recording, and after dissection, intestinal tissues were aseptically collected to measure the development differences of small intestinal villi and crypts, as well as the colonization of lactic acid bacteria and Escherichia coli. The results showed: 1) The microcapsule encapsulation efficiency reached 81.6%, with a particle size of 84.3 m. After treatment in simulated gastric fluid for 120 min, the survival rate of Lactobacillus plantarum was 62.36%, and the survival rate reached 45.9% after storage at 37 ℃ for 120 days. 2) Compared with the PBS group, Lactobacillus plantarum, Lactobacillus plantarum + 0.5% TPPPS, and microcapsules all promoted weight gain in mice, increased the number of lactic acid bacteria in the small intestine, decreased the number of Escherichia coli, and improved the development of small intestinal villi and crypts to varying degrees, with the microcapsule group showing the optimal effect. The results

suggest that the probiotic effect of Lactobacillus plantarum in combination with TPPPS is significantly superior to that of Lactobacillus plantarum alone, and microcapsules prepared from both can further enhance their probiotic effects.

Full Text

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Abstract

As a microecological feed additive, *Lactobacillus plantarum* is vulnerable to adverse factors such as gastric acid, bile acids, and digestive enzymes in the digestive tract after administration. Taishan pine pollen polysaccharide (TPPPS), as a water-soluble plant polysaccharide, is also susceptible to damage by gastric acid and enzymes. To protect the biological activity of *L. plantarum* and TPPPS and explore whether a synergistic effect exists between them, this study prepared microcapsules with *L. plantarum* and TPPPS as core materials, optimized the encapsulation process through orthogonal experiments, and evaluated the properties of microcapsules prepared under optimal conditions. Forty-eight specific-pathogen-free (SPF) mice weighing approximately 20 g were selected and randomly divided into 4 groups (n=12). The four groups were orally administered 1 mL of *L. plantarum*, *L. plantarum* + 0.5% TPPPS, microcapsules, or phosphate-buffered saline (PBS) daily. The feeding trial lasted 42 days. Every week, three mice from each group were randomly selected for weighing and recording. After necropsy, intestinal tissues were aseptically collected to measure differences in small intestinal villus and crypt development and to determine the colonization of lactic acid bacteria and *Escherichia coli*. The results showed: 1) The microcapsule encapsulation efficiency reached 81.6% with a particle size of 84.3 μm. After 120 min treatment in simulated gastric juice, the survival rate of *L. plantarum* was 62.36%, and after 120 days storage at 37 °C, the survival rate reached 45.9%. 2) Compared with the PBS group, *L. plantarum*, *L. plantarum* + 0.5% TPPPS, and microcapsules could promote weight gain in mice to varying degrees, increase the number of lactic acid bacteria in the small intestine, decrease the number of *E. coli*, and improve small intestinal villus and crypt development, with the microcapsule group showing the optimal effects. These results indicate that the probiotic effect of *L. plantarum* combined with TPPPS is significantly superior to *L. plantarum* alone, and microcapsules prepared from the combination can further enhance their beneficial effects.

Key words: *Lactobacillus plantarum*; Taishan pine pollen polysaccharide; microcapsules; small intestinal villi

Introduction

Lactobacillus plantarum is a normal flora in the animal intestine with good intestinal adhesion capacity, capable of producing lactic acid and antibacterial substances [1-3], inhibiting the proliferation of putrefactive and pathogenic bacteria in the intestine, regulating the balance of microbial flora in the animal digestive tract, promoting nutrient digestion and absorption, and enhancing immunity [4-6]. However, live probiotic preparations are susceptible to adverse factors such as low pH gastric acid, bile acids, and digestive enzymes in the digestive tract after entering the animal' s gastrointestinal tract, making it difficult for sufficient viable bacteria to reach or colonize the intestine to exert their effects [7]. Microencapsulation technology utilizes natural or synthetic materials to embed and solidify core substances such as solids, liquids, or gases, which can significantly enhance bacterial tolerance to adverse external environments and greatly improve bacterial survival rates upon entering the intestine [8].

Polysaccharides are biological macromolecules that enhance immunity, and possess antimicrobial, antiviral, and antiparasitic functions [9]. Previous studies in our laboratory have demonstrated that Taishan pine pollen polysaccharide (TPPPS) can significantly enhance animal production performance and immune function [10-11]. However, whether a synergistic effect exists between *L. plantarum* and TPPPS remains unclear. As a water-soluble polysaccharide, TPPPS is susceptible to damage by gastric acid and enzymes. Therefore, this experiment used *L. plantarum* and TPPPS as core materials and sodium alginate (AIg) and chitosan as wall materials to prepare microcapsules. Through orthogonal experiments, the microencapsulation process was optimized to determine the optimal conditions for preparing *L. plantarum*-TPPPS microcapsules. These microcapsules were then orally administered to specific-pathogen-free (SPF) mice to evaluate their effects through production performance measurements, aiming to provide experimental basis for developing novel probiotic microcapsules.

Materials and Methods

1.1 Materials

1.1.1 Strain *Lactobacillus plantarum* 21794 was purchased from the China Center of Industrial Culture Collection.

1.1.2 Reagents Sodium alginate (AIg) was purchased from Xiya Reagent Company; chitosan from Solarbio Company; pepsin from Sigma Company; and trypsin from BioBasic Company.

1.1.3 Instruments Laminar flow hood (Zhejiang Sujing Purification Equipment Co., Ltd.); JJ-2 tissue homogenizer (Wuhan Gelemo Detection Equipment

Co., Ltd.); pH meter (Shanghai Leici Instrument Factory); electric thermostatic incubator (DNP-9082, Shanghai Jinghong Laboratory Equipment Co., Ltd.); optical microscope (55i, Nikon, Japan).

1.2 Preparation and Characterization of Microcapsules

1.2.1 Preparation of *L. plantarum* Suspension The activated strain was inoculated into MRS liquid medium and cultured at 37 °C for 24 h. The cultured bacterial suspension was centrifuged (4,000 r/min, 15 min, 4 °C), the supernatant was discarded, and the bacterial pellet was washed twice with sterile physiological saline. The bacterial cells were then resuspended in physiological saline to achieve a concentration of 10^{10} CFU/mL and stored at 4 °C.

1.2.2 Microcapsule Preparation Process TPPPS was added to the prepared bacterial suspension and mixed uniformly to achieve a TPPPS concentration of 1 mg/mL. Then, sterilized AIg (2.0%) and emulsifier (vegetable oil containing 1.0% Span 80) along with a certain amount of calcium carbonate (dissolved in a small amount of water) were added to the bacterial suspension for emulsification (3,000 r/min, 60 min) to form a water-in-oil emulsion. After complete emulsification, an appropriate amount of glacial acetic acid was added to reduce the reaction pH, and the reaction continued for 30 min. Then, 1.0% Tween 80 solution was added for solidification for 30 min. After standing and centrifugation, the product was washed with physiological saline to obtain AIg gel beads. The AIg gel beads were then poured into 0.4% chitosan solution for cross-linking. After 1 h of solidification, the microcapsules were centrifuged, washed, and stored at 4 °C.

1.2.3 Formulation Screening The volume ratio of aqueous to oil phase (A), mass ratio of calcium carbonate to AIg (B), molar ratio of calcium carbonate to glacial acetic acid (C), and chitosan concentration (D) were selected as influencing factors. A 4-factor, 3-level L9(3^4) orthogonal experimental design was employed to investigate the effects of these four factors on microcapsule encapsulation efficiency. The optimal conditions for preparing *L. plantarum*-TPPPS microcapsules were screened with reference to experimental indicators such as particle size and sphericity. The L9(3^4) orthogonal experimental design for *L. plantarum*-TPPPS microcapsule formulation is shown in Table 1 .

1.2.4 Measurement of Particle Size and Encapsulation Efficiency Microcapsules were placed in decapsulation solution [12] and stirred for 30 min. After complete dissolution, viable bacteria were counted. Encapsulation efficiency (%) = (number of viable bacteria in microcapsules / number of viable bacteria in raw materials) × 100.

Microcapsules were observed under an optical microscope, and particle sizes were measured using a micrometer. More than 500 microcapsules were counted, and the average particle size was calculated according to the following formula:

Dav = Σ(nd)/Σn, where Dav is the average particle size, d is the microcapsule diameter, and n is the number of microcapsules.

1.2.5 Tolerance Test in Simulated Gastric Juice One milliliter of prepared microcapsule sample was placed in 9 mL of simulated gastric juice [12] and oscillated at 37 °C, 180 r/min for 0, 30, 60, 90, and 120 min. Samples were then taken, and viable bacterial counts were determined by gradient dilution method, with unencapsulated bacteria as control.

1.2.6 Storage Stability Test Prepared microcapsule samples were sealed and placed in a 37 °C incubator for 120 days. Every 30 days, 1 mL of microcapsules was sampled to measure viable bacterial counts, with unencapsulated bacteria as the control group.

1.3 Animal Experiment

1.3.1 Experimental Design Forty-eight SPF mice weighing approximately 20 g (female, purchased from Taibang Biological Products Co., Ltd.) were randomly divided into 4 groups (n=12). The mice were housed at 23-28 °C and allowed 3 days to acclimate before the experiment. The four groups were orally administered 1 mL of *L. plantarum* (LP group), *L. plantarum* + 0.5% TPPPS (LP+TPPPS group), microcapsules [Micro(LP+TPPPS) group], or phosphate-buffered saline (PBS group) daily. The feeding trial lasted 42 days, and relevant indicators were sampled and measured on days 7, 14, 21, 28, 35, and 42. Feeding was stopped 12 h before sample collection.

1.3.2 Body Weight Changes and Intestinal Bacterial Colonization Three mice were randomly selected from each group and weighed. After necropsy, ileal tissue samples were aseptically collected, ground, and serially diluted. The dilutions were plated on MRS medium and MacConkey medium to determine viable bacterial counts.

1.3.3 Small Intestine Histological Analysis Three mice from each group were necropsied, and small intestinal tissue samples were aseptically collected to measure villus height, crypt depth, and the villus height/crypt depth ratio.

1.4 Data Processing and Analysis

All data were analyzed using SPSS 17.0 software with Duncan' s multiple comparison test. Data are expressed as mean ± standard deviation, with $P<0.05$ considered statistically significant.

Results

2.1 Optimal Formulation Screening

The optimal microcapsule formulation was screened by measuring encapsulation efficiency and particle size. Using encapsulation efficiency as the experimental indicator, a larger range (R) indicates greater influence of that factor on the indicator. According to the results in Table 2 , the influence of the four factors A, B, C, and D on microcapsule encapsulation efficiency decreased sequentially: aqueous to oil phase volume ratio > calcium carbonate to AIg mass ratio > calcium carbonate to glacial acetic acid molar ratio > chitosan concentration. Data analysis revealed that A2B2C3D2 was the optimal encapsulation condition, though this combination was not initially included in the experimental design, so it was tested separately. The results showed that the A2B2C3D2 combination achieved an encapsulation efficiency of 81.6% and a particle size of 84.3 μm, which was comprehensively superior to other combinations. Therefore, the optimal production process for *L. plantarum* and TPPPS microcapsules was: aqueous to oil phase volume ratio of 1:3, calcium carbonate to AIg mass ratio of 1:4, calcium carbonate to glacial acetic acid molar ratio of 1:4, and chitosan concentration of 4 g/L.

Under optical microscopy, microcapsules prepared under optimal conditions showed regular spherical shape with uniform particle size distribution (Figure 1 [Figure 1: see original paper]). Under high magnification, empty AIg gel beads appeared translucent (Figures 1A, 1D), AIg gel beads encapsulating *L. plantarum* and TPPPS showed reduced transparency (Figures 1B, 1E), and after double coating with AIg and chitosan, the microcapsules appeared darkest with more uniform particle size (Figures 1C, 1F).

2.2 Tolerance to Simulated Gastric Juice

The tolerance of microcapsules to simulated gastric juice was evaluated by detecting the survival rate of microencapsulated *L. plantarum* in simulated gastric juice. As shown in Table 3 , after 30 min of simulated gastric juice treatment, the survival of unencapsulated *L. plantarum* in the control group was significantly lower than that in the microcapsule group ($P<0.05$). After 60 min treatment in simulated gastric juice, the survival rate of the microcapsule group was 70.83%, and after 120 min it was 62.36%, while unencapsulated *L. plantarum* in the control group died completely after 60 min treatment in simulated gastric juice. These results demonstrate that microencapsulation significantly improved the tolerance of *L. plantarum* to simulated gastric juice.

2.3 Storage Stability

As shown in Table 4 , after 120 days storage at 37 °C, the survival rate of *L. plantarum* in microcapsules was 45.9%, while that of unencapsulated *L. plantarum* in the control group was 31.2%, with a significant difference in viable bacterial counts between the two groups ($P<0.05$). These results indicate that the

storage stability of microencapsulated *L. plantarum* was significantly improved.

2.4 Effects on Body Weight and Intestinal Bacteria

As shown in Table 5 , the body weight of mice in the Micro(LP+TPPPS) and LP+TPPPS groups was significantly higher than that in the LP and PBS groups ($P<0.05$), with the Micro(LP+TPPPS) group showing the highest body weight. No significant difference was observed between the LP and PBS groups ($P>0.05$). Compared with the PBS group, the other three groups showed a trend of increased lactic acid bacteria and decreased *E. coli* to varying degrees. The differences between the Micro(LP+TPPPS) and LP+TPPPS groups and the PBS group were significant ($P<0.05$), while the difference between the LP and PBS groups was not significant ($P>0.05$). These results indicate that *L. plantarum* alone can improve the intestinal environment, maintain intestinal flora balance, and improve mouse production performance, with better effects when used synergistically with TPPPS, and the best effects when both were microencapsulated.

2.5 Effects on Small Intestinal Villus Morphology

As shown in Table 6 , villus heights in the Micro(LP+TPPPS), LP+TPPPS, and LP groups were significantly higher than those in the PBS group ($P<0.05$). Crypt depths in the Micro(LP+TPPPS), LP+TPPPS, and LP groups were significantly lower than those in the PBS group ($P<0.05$), with no significant difference between the Micro(LP+TPPPS) and LP+TPPPS groups ($P>0.05$). The villus height/crypt depth ratio in the Micro(LP+TPPPS) and LP+TPPPS groups was significantly higher than that in the PBS group ($P<0.05$), while the LP group was slightly higher than the PBS group but the difference was not significant ($P>0.05$).

Discussion

Probiotics can influence the microecological balance in animals, promote the growth and reproduction of beneficial intestinal bacteria (such as *Bifidobacterium* and *Lactobacillus*), produce various digestive enzymes, and enhance digestive enzyme activity, thereby facilitating the decomposition and utilization of nutrients. However, several factors affect the efficacy of liquid probiotic preparations in clinical use, including high nutritional requirements, slow proliferation rates, susceptibility to the intestinal environment, and difficulty in colonizing the intestine. Research has shown that probiotic intake must reach 10^7 CFU/g to exert health functions [13]. Therefore, providing effective physical barriers for probiotics to resist adverse environments is essential to ensure their beneficial effects. Microencapsulation of bacterial cells, with its advantages of obtaining high-density cell populations, high yield, low contamination, and reusability, has become a research hotspot in bioengineering [14].

Polysaccharides are macromolecular compounds composed of various identical

or different monosaccharides and uronic acids condensed through glycosidic bonds. They naturally exist in various organisms including animals, plants, and microorganisms, and are fundamental substances for maintaining life activities. Many plant polysaccharides as novel additives have been proven to possess multiple biological functions such as antiviral, antitumor, antioxidant, and anti-radiation activities, with characteristics of being non-toxic, harmless, and residue-free. Our laboratory has conducted a series of studies on TPPPS in recent years, finding that TPPPS can significantly improve the production performance of Rex rabbits, enhance immune function in immunosuppressed mice, improve the immune efficacy of rabbit hemorrhagic disease tissue inactivated vaccine, and directly inhibit avian leukosis virus subgroup B (ALV-B). However, the physicochemical properties of polysaccharides are affected by factors such as molecular weight, chemical composition, structure, and conformation, making them susceptible to damage by the gastric environment [15-16]. In practical applications, injection or oral administration methods are cumbersome and involve significant losses, which is not conducive to their application and promotion in production. Therefore, to improve probiotic survival and avoid damage to the biological activity of TPPPS by gastric acid, this experiment co-encapsulated *L. plantarum* and TPPPS into microcapsules and used encapsulation efficiency as an indicator to screen for optimal preparation conditions.

Microcapsules prepared under optimal conditions achieved an encapsulation efficiency of 81.6% with an average particle size of 84.3 μm, good sphericity, uniform size, and strong acid resistance, thereby increasing the number of *L. plantarum* reaching the intestine. Therefore, the microcapsule membrane prepared in this study provided a uniform and stable microenvironment for *L. plantarum*, offering support and protection for the bacteria, enabling long-term storage, and solving the problem of unstable efficacy of *L. plantarum* as a feed additive.

The animal intestinal flora forms a vast and complex microecological balance system that plays an important role in maintaining gastrointestinal health, with nutritional, barrier, and immune functions. After probiotics enter the body, they act as immune adjuvants on lymphoid tissue aggregates with antigen recognition sites in the intestine, activating gut-associated lymphoid tissue and enhancing secretory IgA antibody secretion, thereby resisting colonization of exogenous harmful microorganisms in the intestine and forming a biological barrier [17]. Through analysis of intestinal bacterial colonization in mice, this experiment found that the combination of *L. plantarum* and TPPPS was significantly superior to *L. plantarum* alone in effectively promoting the proliferation of lactic acid bacteria and inhibiting the proliferation of *E. coli* in the mouse intestine. This demonstrates that the combined use of *L. plantarum* and TPPPS has a synergistic effect that can cooperatively improve the intestinal environment.

The small intestine is the primary site for nutrient digestion and absorption in the body, and intestinal health plays a crucial role in the digestion, absorption, and transport of nutrients [18]. Increased villus height enlarges the surface area for nutrient absorption in the small intestine, so villus length directly affects an-

imal growth and development [19]. Crypt depth mainly reflects the generation rate of epithelial cells, while the villus height/crypt depth ratio comprehensively reflects small intestinal functional status. A decreased ratio indicates reduced digestive and absorptive function, affecting nutrient absorption and decreasing animal production performance [20]. Through analysis of morphological changes in mouse small intestine, this experiment found that *L. plantarum*, *L. plantarum* + 0.5% TPPPS, and microcapsules could increase small intestinal villus height, decrease crypt depth, and increase their ratio to varying degrees. Moreover, the synergistic effect of *L. plantarum* with TPPPS was superior to *L. plantarum* alone, with the best effects observed after microencapsulation. Additionally, this experiment found that the combination of *L. plantarum* and TPPPS could promote weight gain in mice, with effects significantly superior to *L. plantarum* alone. These results indicate that the combination of *L. plantarum* and TPPPS has synergistic effects in promoting weight gain, improving intestinal environment, and intestinal development in mice, similar to reports by Wang Yongfen et al. [21-22] that Astragalus polysaccharide combined with probiotics showed significantly better probiotic effects in chickens than probiotics or polysaccharides alone. These findings suggest that *L. plantarum* and TPPPS can improve intestinal mucosal development and regulate intestinal microbial composition, thereby promoting mouse growth and development.

The *L. plantarum*-TPPPS microcapsules prepared in this experiment exhibited uniform particle size, high encapsulation efficiency, tolerance to gastric acid environment, and long shelf life. Moreover, these microcapsules significantly improved intestinal development and microecological environment in mice and promoted growth, with effects significantly superior to *L. plantarum* alone.

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Note: Figure translations are in progress. See original paper for figures.