Preparation and *in vitro* characterization of *Lactobacillus plantarum* and *Lactobacillus fermentum* beads using low methoxyl pectin

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**Abstract**

*Lactobacillus plantarum* (*LP*) and *Lactobacillus fermentum* (*LF*) were encapsulated into low methoxyl pectin (*LMP*) beads by CaCl₂ external ionization. The encapsulating efficiency (*EE*), *in vitro* release and stability studies were performed by plate counting. The results showed that more than 99.99% *EE* were obtained. The contents of *LF* and *LP* were 10¹¹ cfu/g beads (dry base). Stability study showed that encapsulation improved the stability under ambient temperature compared with the unencapsulated microbes. It provides the foundation for the further development and application of composite *Lactobacillus* beads in modulation of intestinal and systematic function.

**Keywords:** Low methoxyl pectin Bead; *Lactobacillus plantarum*; *Lactobacillus fermentum*

**INTRODUCTION**
Probiotics is a kind of live microorganisms which can exert the health effects when there are enough number reaching colon or the lower parts of small intestine [1-4], and have been studied extensively for their use as new therapy to the treatment of many diseases, including the cardiovascular disease, obesity and its related syndrome. Studies also found that the intestinal flora were most abundant in the colon, therefore, it is conceivable that the changes of colon flora composition for weight loss may have a more direct effect. However, because of the instable nature of some probiotics during passage into gastrointestinal (GI) system where extreme pH and bile salt exist, many measures have been taken, of which microencapsulation technique showed great promise [5-15]. However, because of the intestinal soluble features of some materials such as alginate [16, 17], more efforts have been directed to find more sable materials for probiotics encapsulation.

Pectin is plant-derived component which has been used in many areas based on its different characters [18-22]. According to the methoxyl contents, pectin can be divided into high methoxyl pectin (HMP) and low methoxyl pectin (LMP). In recent years, LMP has been studied in drug delivery system as it can form beads under calcium chloride treatment [22-27], which are widely used as colon delivery carrier [28-30]. In this study, the LMP was used for encapsulating LF and LP individually or in combination. The in vitro tests were performed to verify the possibility of using this kind of material as carrier for colon-targeted probiotic delivery system.

MATERIALS AND METHODS

Strain

*Lactobacillus fermentum subsp. bulgaricus* (LF, CGMCC1.1880) was purchased from China General Microbiological Culture Collection Center (CGMCC). *Lactobacillus plantarum* (LP) was isolated from Northeast sauerkraut in our lab.

Preparation of cells for encapsulation

The frozen cultures of *Lactobacillus fermentum subsp. bulgaricus* (LF) and *Lactobacillus plantarum* (LP) were transferred independently into MRS broth at 4% (v/v) under aerobic environment. Then the cultures were harvested after centrifugation at 4000rpm (6C type low speed centrifugator, Labnet, internation. inc) for 15 min. The pellets were subcultured 3 passages and collected at 4000rpm for 15 min.
Low methoxyl pectin (food grade, Anhui Yu Ning Biotechnology Co. Ltd.) beads containing LP and LF alone or in combination at 1:1 (v/v) were prepared by external ionotropic gelation [20]. In brief, 2% pectin aqueous solution containing LP and LF alone or in combination was extruded into 300mM anhydrous calcium chloride (food grade, Anhui Yu Ning Biotechnology Co. Ltd.) solution using 10 mL injector with 7# needle under stirring. After extrusion, the solution was stirred for another 20 minutes. After that, the beads were collected by filtration and freeze-dried after mixing with cryoprotectant. The formula for cryoprotectant was composed of 10.43 g skim milk (Inner Mongolia Yili Industrial Group Limited by Share Ltd), 8.91g sucrose and 0.23g MnSO₄ in 100mL formulation base, which were mixed well and boiled at 100 °C for 10 min, then cooled down to 4 °C. The beads were mixed with cryoprotectant at 4:1 (w/v) [31]. Then the mixture was prefrozen at -20 °C for 3 h, then freeze dried for 12 hours. The freeze drying products were collected after dried. For storage, these products were subdivided into three parts, one was stored in desiccator at room temperature; the other parts were stored at 4 °C and -20 °C.

The supernatants were collected and used for enumeration of the LP and LF by plate counting as follows. The morphology of the beads was characterized by optical microscopy.

**Encapsulation efficiency (EE)**

The EE was calculated by plate counting using MRS agar. The MRS agar medium was prepared according to manufacturer’s guide and autoclaved at 121 °C for 20 min using LDZX-50KB type vertical pressure steam sterilizer (Shanghai Shen An medical instrument factory). After cool down to 50 °C, the medium was poured onto 10 cm plates and continually cool down. Then the collected flora was diluted serially and spread onto the plates. After anaerobically culture for 40-44h, the LP and LF were counted and then calculated as cfu/mL (for supernatants) or cfu/g (for beads). For enumeration of the LP and LF in supernatants, the supernatant was diluted directly and spread on pates.

The EE was calculated according to Reddy et al [32] based on plate counting technique with some modifications.

$$\text{EE}=\log (A-B)/ \log A*100\%$$

Where A and B represent the total number of Lactobacillus added (CFU/ mL) and those in supernatants.

The total number of lactobacillus was counted by plate counting also. In brief, the LP and LF
cultures were spread onto MRS plates after collection and dilution into suitable concentrations.

**In vitro release**

Simulated gastric fluid (SGF) was prepared according to the U.S. Pharmacopeia [33], with some minor modifications. In brief, 100mL SGF containing 1.00 g pepsin (Tissue Culture Grade, AMERCO), the pH was adjusted to 2.0 with 10 N HCl. Simulated bile fluid (SBF): 1% SBF was prepared by adding 1g No. 7 bile salt into 99 mL sterile water. Simulated intestinal fluid (SIF): 1g pancreatin (Tissue Culture Grade, AMERCO) was dissolved into 100mL 0.2M PBS (pH 6.8) before use.

*In vitro* release tests were performed by putting the beads in SGF for 2h, SBF 20min, SIF 2h, continually [34]. After each process, the beads were collected and disintegrated using 50mM Ethylene diamine tetra-acetic acid (EDTA) solution as above, then washed with autoclaved saline. The collected flora was spread on 10 cm MRS plates for enumeration in duplicate.

**Stability study**

The freeze drying beads were stored at -20°C, 4°C and ambient temperature in desiccator, respectively, samples were tested weekly in triplicate by plate counting as above.

**Determination of microbe number in beads**

*Strain specific primer design*  Bacterial DNA was extracted using Ezup pillar bacterial genome DNA extraction kit. The LF and LP were amplified using 16S rDNA sequences, then subjected to sequence analysis. The individual specific sites were selected for primer design using Primer 5.0 primer design software from NCBI website.

**RT-PCR**  The quantification of the LP and LF was based on the pure culture standards. A tenfold dilution series of the strain in MRS was prepared corresponding to $10^4$ to $10^8$ CFU/mL LP and LF, respectively. These dilutions were used for RT-PCR analysis.

Real-time PCR was performed by Bio-Rad apparatus. 0.1g Lyophilized composite Lactobacillus beads were taken out and subjected to 50 mmol/L EDTA solution for thorough breakdown. RT-PCR quantitative analysis of the LP and LF was performed using double-stranded chimeric SYBR dye method.

The specific primers of LP and LF were designed as follows:

**LF**: PrimerF CAACAAGGGAAAAACGCGCGA
Amplification was carried out in a 20 uL final volume containing 1 uL bacterial DNA as templates. 2.0 uL 10×PCR buffer, 1.6 uL 2.5mM dNTPs, 1.2 uL 2.5mM Mg\(^{2+}\), 0.2 uL Taq DNase, 1.0 uL former primer, 1.0 uL reverse primer and 12 uL ddH\(_2\)O. The thermocycler programs had denaturation at 94°C for 5 min, then 35 cycles with the following steps: denaturation at 94°C for 1 min, annealing at 53°C for 30 s (for LP) or 55°C for 30 s (for LF), and elongation at 72°C for 10 min. For each step, the temperature transition rate was 20 °C. Fluorescence signal was detected at the end of the annealing step in every cycle.

**Statistical analysis**

The results were expressed as mean ±std. All the data were analyzed using Origin 8.0 software. Comparisons were analyzed by one-way analysis of ANOVA.

**RESULTS AND DISCUSSION**

**In vitro characterization of the beads**

The study is aimed at the characterization of LMP beads loading LP and LF alone or in combination in vitro/in vivo. Figure 1 showed the morphology of the beads which were round and smooth in solutions with excellent disperse character. The average sizes were 2.00±0.02mm. After freeze drying, the beads were light yellow. Our results showed the spherical morphology with the size which are suitable for animal experiments. The size distribution may be determined by several factors, such as the internal diameter of syringe needle, the pectin concentration, the stirring rate. In this study, the optimized protocol documented by Jung et al was used [35]. There was 3 to 4 log cfu decrease of the LP and LF in supernatants from beads prepared with LMP encapsulation, indicating that EE was higher than 99.9%.

Table 1 showed the survivability after in vitro release profile in different simulated fluids. All the unencapsulated strains showed significantly decrease in activities after subjected to SGF, with 3.83, 3.95, 3.74 log CFU reduction for LF+LP, LF and LP, respectively. After subjected to SBF treatment, there is another 1 log CFU reduction, but all were relatively stable in SIF.
In comparison, the LF+LP, LF and LP beads showed only 1.10, 1.10, and 0.87 log CFU reduction after SGF treatment. Another 0.78, 0.69, 1.04 log CFU reduction appeared after SBF treatment. Similar to unencapsulated strains, all were stable in SIF, indicating that it can reach small intestine or colon only if the higher survival rate can be obtained upon passage through gastric acid or bile salt.

**Stability analysis**

Stability of the probiotics during storage is another important consideration as this affect the applicability of the products. Generally, temperature, light, oxygen and water can directly affect the stability of the encapsulated probiotics [36]. For the beads after freeze drying, the results have been obtained for the beads prepared in different time, which showed that more stability was found for those stored at -20 and 4°C. Encapsulation with LMP did not change the stability of the microbes (Fig. 2).

**Quantification of the strains in beads**

The strain-specific real-time PCR was conducted to quantify the number of the LP and LF in beads. The PCR products of LF and LP were imaged by agar gel electrophoresis (Fig. 3), then sequenced and checked for the accuracy of the segments using web tool of the National Center of Biotechnology Information (NCBI) Blast software (data not shown).

The qPCR standard curve was plotted by diluting the LF and LP cultures from 10⁴ CFU to 10⁸ CFU/g. Amplification efficiency were 95% for both strains (Fig. 4). Using the individual standard regression equation, the number of LP and LF in beads were absolutely calculated as 10¹¹ cfu/g bead (dry base). Fig. 5 showed the absolutely quantification curves of the LP and LF in beads. The accurate number of each probiotics in beads is important as this will enable us to design the composition of compound probiotics beads.

In summary, the external gelation by calcium chloride is ideal for encapsulation of LP and LF into LMP. The compound beads showed good stability in different storage conditions.

**ACKNOWLEDGMENTS**

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Figures and Tables

Fig. 1 image of LMP beads. Left: LMP beads before freeze drying; Right: LMP beads with protectant after freeze drying
Fig. 2 Stability studies on the freeze-dried microcapsules storage under 4°C (A), 20°C (B) and -20°C (C), respectively. All the viable flora number was calculated as the number of lactobacillus on dry base (CFU/g) for comparison. LP, Lactobacillus plantarum; LF, Lactobacillus fermentum; MC, microcapsule; UM, unmicrocapsulated
Figure 3  The gel electrophoresis image of Lactobacillus fermentum and plantarum PCR products

Fig. 4  qPCR standard curve for quantification of LF and LP, the LP and LF cultures were diluted serially from 10^4 CFU to 10^8 CFU/g.
Fig 5 The absolute quantitative graphs of Lactobacillus fermentium (A) and Lactobacillus plantarum (B) in microcapsules
Table 1 *In vitro* simulated gastric-intestinal tract test of the beads (log CFU)

<table>
<thead>
<tr>
<th></th>
<th>LF+LP</th>
<th>UM</th>
<th>LF+LP MC</th>
<th>UM LF</th>
<th>MC</th>
<th>LF</th>
<th>UM</th>
<th>LP</th>
<th>MC</th>
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<tr>
<td>Original</td>
<td>10.54±0.07</td>
<td>10.32±0.06</td>
<td>10.52±0.05</td>
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<td>SGF 2h</td>
<td>6.71±0.12</td>
<td>9.22±0.10</td>
<td>6.57±0.10</td>
<td>9.21±0.11</td>
<td>6.73±0.08</td>
<td>9.34±0.09</td>
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<td>SBF 20min</td>
<td>5.66±0.21</td>
<td>8.54±0.17</td>
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<tr>
<td>SIF 3h</td>
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<td>8.56±0.16</td>
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Note: SGF, simulated gastric fluid; SBF, simulated bile fluid; SIF, simulated intestine fluid. SGF-SBF-SIF refers to the LB underwent the SGF treatment for 2h, SBF for 20min and SIF for 3h. Different lowercases and uppercases represent the significant difference at p<0.05 and p<0.01 level in the same column, respectively. All the flora were counted on dry lactobacillus base. LP, Lactobacillus *plantarum*; LF, Lactobacillus *fermentum*; MC, microcapsule; UM, unmicrocapsulated

REFERENCES


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