Preparation and Properties of a Novel Sodium Alginate Microcapsule

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Abstract. In this study, sodium alginate was used as the main embedding material, and sodium polyacrylate had been immobilized on sodium alginate with calcium ions as crosslinking agent for preparing a new type of lactic acid bacteria entrapment material. The aim of this study was to investigate the viability of Lactococcus lactis NCU1315 under simulated gastrointestinal conditions and the physical properties of the microcapsules and their protective effects on Lactobacillus to develop a feasible and stable method for preparing microbial particles. The hardness of Alg-1 and Alg-2 microcapsules varied with time in simulated gastric juice and showed significant difference at the second and third hour (P<0.05, P<0.01). The particle size of Alg-1 and Alg-2 microcapsules varied with time in simulated gastric juice and showed significant difference at the second and third hour (P<0.05, P<0.01). Alg-1 had no significant effect on the growth of NCU1315, but Alg-2 had inhibitory effect on the growth of Lactococcus lactis NCU1315. The survival rate of Lactococcus lactis in Alg-1 and Alg-2 microencapsulated groups was significantly higher than that in Free cell group at 24h, 48h and 72h (P<0.05, P<0.01). However, the survival rate of Lactococcus lactis in Alg-1 and Alg-2 microencapsulated groups decreased with time (P<0.05, P<0.01).

1. Introduction
Lactic acid bacteria (LAB) is a type of bacterium that can produce lactic acid by fermentation with lactose or glucose carbohydrates. LAB lives in animals or the intestinal tract, which promotes metabolism in the body. It is a kind of probiotics [1]. Abnormal temperature, humidity, pH and other conditions experienced in the processing, sterilization, preservation and other complicated steps of the lactic acid bacteria products seriously reduce the activity of Lactobacillus [2]. At the same time, in the process of oral administration of LAB, the user's stomach is extremely acidic, which will seriously reduce the activity of probiotics before reaching the intestine, thus making the effect and function of Lactobacillus difficult to play [3]. Therefore, it is a great challenge to preserve the lactic acid bacteria in the production process.

In terms of preservation, microencapsulation can be used to improve the survival rate of probiotics in processing and storage, especially in gastric acid transport. The process uniformly coats the functional ingredients with food grade and biodegradable materials, separating the internal phase from the surrounding matrix, which can be used for lactic acid bacteria for several months or years [4]. At the same time, it helps to separate the core material from its environment until it is released, thereby increasing its stability, extending the shelf life of the core, and providing a sustained and controlled
release that masks the taste, color or odor [5]. Microencapsulation technology is one of the most promising bacterial retention technologies. Many researchers use microencapsulation technology to study the protective effects of probiotics.

Probiotic cell concentrations usually need to be stored for a longer period of time before food production and ingestion, and are not affected by the gastric acid transport process [6]. Probiotics are encapsulated in a gel matrix of biomaterials such as alginate, k-carrageenan, gellanous/xanthan gum, while in freeze-drying, spray-drying, etc., microorganisms are completely released into the product [7]. However, few materials have been studied to improve the performance of alginate microcapsules. The aim of this work was to investigate the viability of lactobacillus under simulated gastrointestinal conditions and the physical properties of the microcapsules and their protective effects on lactobacillus to develop a feasible and stable method for preparing microbial particles. It is used in food and medical applications. The cytotoxicity of Alg-1 and Alg-2 microcapsules were not detected cytotoxicity.

2. Materials and methods

2.1. Materials

Lactococcus lactis NCU1315 (Chengdu Institute of Microbiology, Chinese). It is stored in a liquid nitrogen tank and used after 3 generations of resuscitation culture. The cells were cultured in MRS medium and cultured in MRS broth medium. For preparation of the cell banks, the cells were grown in MRS broth at 37 °C for approximately 15h. The strains were collected at 4500 r/min, centrifuged for 10 min, suspended in 0.9% physiological saline, and inoculated with a suspension at about 10^6 cfu/mL.

Mouse fibroblast L929 (Shanghai Yaji Biotechnology Co., Ltd. Chinese), and the number of stable passages were 10-15 times. Culture method: 90% DMEM medium (GIBCO, added NaHCO₃ 1.5g/L) 10% fetal bovine serum, 1%-5% cellbio double antibody.

2.2. Methods

2.2.1. Preparation of sample. The alginate was dissolved in distilled water (alginate/distilled water=1/50 is Alg-1; alginate/distilled water=1/100 is Alg-2) and stirred with a magnetic stirrer for 10 min in order to completely dissolved, and heated it to about 60 °C under nitrogen gas protected and continue stirring for 40min. Adding 0.18 g of ammonium persulfate initiator into suspensions, and initiate at 58 °C for 15 min. The mixture was neutralized by 4 mol / L sodium hydroxide solution and 1.44 g of the re-distilled acrylic monomer under low temperature conditions (0-4 °C), and then added dropwise to the sodium alginate solution prepared above. The new alginate material was prepared by heating to 75 ℃, protecting reaction with nitrogen gas for 3 hours, adjusting pH to 6.8, dialyzing in distilled water for 3 days, and freeze drying.

2.2.2. Bacterial embedding. The alginate was mixed with the new alginate material. The ratio of alginate/new alginate material was 2% and 1%. Adding 0.3 mol / L of alginate into calcium chloride solution for solidifying at 4 °C for 1 h, then transferred to 0.5% chitosan solution and coated for 20 min. The new alginate microcapsules were obtained by repeated cleaning with sterile ultra-pure water. The well-grown Lactococcus lactis NCU 1315 was collected by centrifugation and dispersed in the above mixed solution.

2.2.3. Microcapsule physical properties. External simulation of gastric fluid. 4 g of pepsin and 2 g of sodium chloride (injection grade) were weighed and placed in 1 L acetic acid solution (0.5 M), stirred with a magnetic stirrer for 15 min to completely dissolve, and suction-filtered through a 0.22 um filter. Observe and record the particle size and hardnes of the new materials after the new microcapsules 1, 2 and 3h.

2.2.4. Effect of new microcapsule materials on the growth of Lactococcus lactis NCU1315. A new alginate material was added to the MRS medium to give a final mass fraction of 1% and 2%, inoculated
with *Lactococcus lactis NCU1315*, cultured at 37 °C for 10 h, and the OD value of the bacterial content was measured by a growth curve analyzer. A blank reference was made in the MRS medium and a growth curve was drawn.

2.2.5. *Microcapsule cytotoxicity test*. Sample leaching: After the Alg-1 and Alg-1 microcapsules prepared in this experiment were irradiated and sterilized, 1 g was weighed in 10 ml of DMEM medium, and extracted in a constant temperature shaking incubator at 90 r/min and extracted at 37 °C for 48 h, suction filtration through a 0.22 μm pore size filter.

Sample grouping: DMEM culture medium diluted with Alg-1 and Alg-1 unembedded *Lactococcus* bacteria NCU1315 sample was diluted to 50%, 25%, 12.5% and then used. 100% medium + 6.4% phenol (positive control): 1.872 mL of medium + 0.128 mL of phenol. 100% medium (negative control): 2 mL medium preparation of MTT--5mg/mL: Weigh 5mg of MTT, dissolved it in 1ml of PBS, filter it with 0.22um filter to remove bacteria, wrap it in tin foil paper and store it at 4°C in the dark.

Cytotoxicity assay: Mouse fibroblasts (L929) were cultured to an exponential growth phase and diluted to a density of 1×10⁵ cells/mL, and added to a 96-well plate at 100 uL per well, with 6 parallel wells per concentration.

Detection: Fibroblast cell was cultured in 5% carbon dioxide incubator at 37 °C for 24 hours and sucked out the medium, adding the above diluent to culture for 48 hours, adding 10 μL of MTT (5 mg/mL) solution to react with cells in dark to incubate for 4 hours at 37 °C, then washed it and added to DMSO, 200ul / well, shaking it horizontally for 10 minutes to stop the reaction, and use an enzyme scale to detect the absorbance value OD₄₉₀ nm. In the negative control group, the normal cells were cultured without other extracts. For specific experimental steps, refer to the latest version of ISO10993 regulations on cytotoxic testing [8].

Formula for Relative proliferation rate (RPR) equation (1).

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RPR = \frac{\text{The experimental group OD}_{490\text{nm}}}{\text{The reference group OD}_{490\text{nm}}} \times 100\%
\]

**Table 1. cytotoxic grading.**

| Cytotoxic grading | Relative appreciation rate | Cytotoxicity          |
|-------------------|----------------------------|-----------------------|
| Grade0            | ≥100                       | No cytotoxicity       |
| Grade1            | 80~99                      | mild cytotoxicity     |
| Grade2            | 50~79                      | Moderate cytotoxicity |
| Grade3            | 30~49                      | severe cytotoxicity   |
| Grade4            | 0~29                       |                       |

2.2.6. *Study on the application of Lactococcus lactis NCU1315 in the gastrointestinal tract of SD rats.* Twenty SPF grade SD male rats weighing (190-200) ±5 g. The rats were housed in an air-conditioned room at the temperature of 23 ± 3 °C and the humidity of 40-65%. During the feeding period, sufficient food and water were given daily. The resistant fluorescent protein gene was constructed and transferred into *Lactococcus lactis NCU1315* and embedded in the new microcapsule materials Alg-1 and Alg-2, and perfused into the rat stomach by gavage. The rats were dissected at 24, 48 and 72 hours after gavage, and the intestines and rectum were taken back and observed with a fluorescence microscope to count the resistant plates.

3. Results

3.1 Physical changes

At present, the embedding methods of *lactic acid bacteria* microcapsules are mainly extrusion, emulsification and spray drying. In this experiment, the extrusion method can encapsulate the active material into the carrier material, and the bacteria has high survival rate and it is suitable for mass
production. The results of the change in particle size and hardness of microcapsules in simulated gastric fluid over time in this study are as follows (see Table 2, Table 3).

The hardness of Alg-1 and Alg-2 microcapsules in the simulated gastric juice changed with time and showed significant difference after the 2 and 3 hours (P<0.05, P<0.01). The changes in particle size of Alg-1 and Alg-2 microcapsules in simulated gastric fluid over time showed significant differences after the 2 and 3 hours (P<0.05, P<0.01). The hardness and particle size of the new microcapsules changed significantly in the simulated gastric juice for 3 hours, but it did not affect the use effect in practical applications.

Table 2. The hardness changes of the new microcapsule in simulated gastric fluid.

| Microcapsule | N  | Original quality (g a) | 1h (g a) | 2h (g a) | 3h (g a) |
|--------------|----|------------------------|----------|----------|----------|
| Alg-1        | 10 | 150.3±5.34             | 178.78±6.38 | 190.78±8.9* | 199.78±7.19** |
| Alg-2        | 10 | 157.4±6.37             | 189.12±6.93 | 221.12±8.56* | 230.12±9.46** |

Note: a: Testing of texture devices. Compared with the original mass *P<0.05, **P<0.01

Table 3. The particle size length of the new microcapsule in simulated gastric fluid.

| Microcapsule | N  | Original length (mm³) | 1h (mm³) | 2h (mm³) | 3h (mm³) |
|--------------|----|-----------------------|----------|----------|----------|
| Alg-1        | 10 | 1.48±0.09             | 1.50±0.13 | 1.52±0.14* | 1.55±0.25** |
| Alg-2        | 10 | 1.47±0.11             | 1.48±0.12 | 1.50±0.09* | 1.54±0.16* |

Note: a: Vernier caliper Measurement. Compared with the particle size length *P<0.05, **P<0.01

3.2. Effects of New Materials on the Growth of Lactococcus lactisNCU1315

The microcapsules are intimately contacted after the encapsulation of Lactococcus lactis NCU1315, and the microcapsules need to pass through the intestinal tract for several hours before releasing the lactic acid bacteria embedded therein. Therefore, it is important to study whether the effect of microcapsule materials on lactic acid bacteria has good affinity. The growth curve of Lactococcus lactis was studied by adding new alginate microcapsules to the MRS (see figure 1). The addition of 1% microcapsule with new material Alg-1 had no significant effect on the growth of Lactococcus lactis NCU1315, while the addition of 2% microcapsule with new material Alg-2 had a significant inhibitory effect on the growth of Lactococcus lactis NCU1315. It indicates that the addition of high concentration microcapsules will affect the growth of Lactococcus lactis NCU1315. However, the right amount of microcapsule new materials will not affect the activity of Lactococcus lactis NCU1315.
Figure 1. Growth curve of *lactococcus lactis NCU1315* with new Alg material.

Note: Alg-free is no microcapsules material for adding into MRS;
Alg-1-1 is a new material for adding 1% Alg-1 microcapsules;
Alg-1-2 is a new material for adding 1% Alg-2 microcapsules;
Alg-2-1 is a new material for adding 2% Alg-1 microcapsules;
Alg-2-2 is a new material for adding 2% Alg-2 microcapsules.

3.3. cytotoxicity

The new microcapsules are used for oral administration and will enter the human body to be in close contact with the cells of the gastrointestinal tissues. If the product is cytotoxic, it will bring health risks to the user and endanger human health. Therefore, this study needs to determine that the product is not cytotoxic for further development and application. The L929 cell line is the most commonly used method for detecting cytotoxicity studies in biomedical materials. During the experiment, the cells adhered well after 48 h of cell plating, and the cells were transparent and full mainly in the form of spindle-shaped growth. The relative proliferation rate results in different gradient extracts are shown in figure 2.

In this experiment, the negative control was 100% DMEM medium, and the positive control added appropriate amount of phenol to the DMEM medium. According to the cytotoxicity classification table 1, the relative proliferation rate of cells in positive control group was between 30 and 49%, which was grade and severe cytotoxicity, so the positive control experiment in this experiment was established. The relative proliferation rates of cells in the 25%, 50% and 100% extracts of Alg-1 and Alg-2 microcapsules were all greater than 100% and were non-cytotoxic. The experimental results show that the new Alg microcapsules prepared in this laboratory are non-cytotoxic and safe to eat.
Figure 2. Cytotoxicity of microcapsule extracts.

3.4. Study results of embedding Lactococcus lactis in the intestine of SD rats

3.4.1. Survival in the rat ileum. The study on the ileum of SD rats by Lactococcus lactis can directly study the use effect of the new Alg microcapsule-embedded Lactococcus lactis. The experimental results are shown in figure 3. Alg-1 and Alg-2 microencapsulated Lactococcus lactis group decreased with time after 24h, 48 and 72h (P<0.01), but the survival rate of Lactococcus lactis was significantly higher than that of free cell. It shows that the activity of the new Alg microcapsules against Lactococcus lactis has a protective effect in the ileum.

Figure 3. Survival of lactococcus lactis NCU1315 embedded in Alg new material in the ileum of rats.

Note: Compared with free cell group *P<0.05, **P<0.01

3.4.2. Survival in the rat rectum. The research on the rectum of Lactococcus lactis in SD rats can directly study the use effect of the new Alg microcapsule-embedded Lactococcus lactis. The experimental results are shown in figure 4. Alg-1 and Alg-2 microencapsulated Lactococcus lactis group decreased with time after 24h, 48 and 72h (P<0.05, P<0.01), but the survival rate of Lactococcus lactis was significantly higher. Free cell group. It shows that the activity of the new Alg microcapsules against Lactococcus lactis has a protective effect in the rectum.
Figure 4. Survival of *lactococcus lactis* NCU1315 embedded in Alg new material in the rectum of rats.

Note: Compared with Free cell group *P<0.05, **P<0.01

4. Discussion

Lactic acid bacteria live in the gut of animals or humans to promote metabolism in the body, which contribute to sensory properties and antiseptic effects. In this laboratory, we used *Lactococcus lactis NCU1315* as a source of bacteria to study the effect of new alginate microcapsule on Lactobacillus. In the process of oral Lactobacillus, the user's stomach is in extremely acidic environment, which will seriously reduce the activity of probiotics before reaching the intestine, so that the effect and function of Lactobacillus are difficult to play [2][9]. Related studies have shown that encapsulated sodium alginate microcapsules provide protection to bacteria while increasing the amount of survivability delivered. In order to successfully encapsulate live bacteria, it is important to maintain the viability of the bacteria during the embedding process and compatibility with the type of drug encapsulation material [10].

Microcapsule embedding technology has many advantages, such as the powerful capsule can withstand months or years of continuous use without damaging cell activity or capsule characteristics, capsule size, large-scale production and economic feasibility of the capsule [11]. Therefore, this experiment uses microencapsulation technology to study the protective effect on probiotics, using sodium alginate as the main embedding raw material, and using sodium polyacrylate to crosslink with calcium ions to link sodium polyacrylate to sodium alginate. A novel lactic acid bacterium embedding material of sodium alginate was prepared.

At present, in domestic and foreign research, microcapsule embedding methods mainly include spray drying, spray cooling or spray cooling, extrusion coating, fluidized bed coating, liposome encapsulation, lyophilization, coagulation, and centrifugal suspension. The active material can be encapsulated into the support material by methods such as separation, co-crystallization, encapsulation, and thermal gel [12]. The physical and chemical properties of the core material or coating material and its application in food and drug substrates affect the choice of packaging process. Extrusion, emulsification, and spray cooling are all effective methods of laboratory packaging in all technologies. Extrusion is an ancient and commonly used method that uses a simple, low-cost method to produce hydrocolloid capsules that minimizes damage to probiotic cells while maintaining high viability [13]. However, it is time consuming and labor intensive in production, and therefore, the method is not suitable for mass production. The emulsion method is a widely used chemical method for encapsulating living cells with hydrocolloids (alginate, carrageenan and pectin) as cell wall materials, usually recovered by membrane filtration techniques. The diameter of the microspheres during the preparation of the microcapsules is affected by the concentration, viscosity and stirring rate of the hydrocolloid solution, and the process is unstable [14].

In the process of embedding the strain, the spray cooling method is adopted in the experiment. The
spray cooling method is a method based on the spray drying method, and the homogenized carrier material is atomized into a dry state during the embedding process. Gas, mainly controlled by product feed, gas flow and temperature, delivers cold air in a hot chamber to make a capsule as a dry powder. This method is one of the commonly used methods of probiotics [3]. Related experimental studies have proved that the spray cooling method has been effectively applied in Bifidobacterium sputum. Related studies compared the survival rates of sodium alginate, chitosan and hydroxypropyl methylcellulose (HPMC) spray-cooled Lactobacillus acidophilus, and found that sodium alginate and HPMC had no effect on cell viability. Chitosan enhanced the storage stability of Lactobacillus acidophilus and recorded a higher viable count after storage for 35 days [15]. Spray drying of Bifidobacterium lactis Bb-12 in whey protein can improve cell viability and 12-week stability under bile conditions. Add thermal protection agent such as trehalose before spray drying, plus starch and soluble fiber to improve storage. The vitality of the culture during the period [16]. The inulin-sodium alginate-skim milk (ISA) capsule is prepared by freeze-drying to increase the vigor of the plant and the function of the probiotic. In this experiment, the hardness and particle size of the new microcapsules after 3 hours in the simulated gastric juice were significantly changed, which ensured that the lactic acid bacteria embedded in the intestinal tract were released after taking the microcapsules for 3 hours, satisfying the actual use effect. In the cytotoxicity of this study, it was shown that the addition of a suitable amount of new microcapsule material did not affect the activity of Lactococcus lactis NCU1315.

Alginic acid is a widely used biopolymer package derived from brown algae (algae), which mainly produces β-D-mannuronic and α-L-guluronic acids. Calcium alginate gelation occurs by cross-linking of Ca²⁺ gel ions with calcium alginate, which is ideal for encapsulation of probiotics at 0.5-4% [17]. Alginate is widely used because of its simple structure, good biocompatibility, low cost, non-toxicity, easy formation of gelatinous substances around bacterial cells and mild processing conditions [18]. Expanding the difficulty of this process and porous particles are major defects in protecting bacteria from the environment [19]. In order to fill this shortcoming, sodium alginate was used as the main embedding material. The sodium polyacrylate was linked to sodium alginate by using sodium polyacrylate and calcium ion cross-linking to prepare a new type of lactic acid bacteria embedding material. Related studies at home and abroad have shown that alginate can be structurally modified by the combination of alginate with other polymers or with other compound capsule coatings or with different additives [20]. In this experiment, no cytotoxicity was detected in the groups of algin-alkali Alg-1 and Alg-2 microcapsules added with different ratios. At the same time, the results of the study of the encapsulation of Lactococcus lactis NCU1315 in the intestine of SD rats showed that the activity of the new Alg microcapsules on Lactococcus lactis has protective effects in the ileum and rectum.

5. Conclusion
In this study, semi-natural macromolecule alginate microcapsules with good affinity to lactic acid bacteria were synthesized in this study. The microcapsules have good physical properties, can improve the survival rate of lactic acid bacteria and provide effective protection for lactic acid bacteria. At the same time, they are non-cytotoxic and have good acid resistance, so they are a good new material for embedding and protecting lactic acid bacteria.

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