Chapter 14

Microencapsulation of Probiotics

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Abstract

Probiotics are susceptible to factors such as stomach acid, enzymes, and bile salts. Also, when incorporated into food matrices, intrinsic or processing factors like low pH, high water activity, or high cooking temperatures can negatively affect the viability of microorganisms. Encapsulation technology can ensure the safe delivery of probiotics to the gut and better survival during processing and storage. Several techniques are used to protect probiotics, for example, emulsion, extrusion, spray-drying, freeze-drying, liposome, electrospinning, and others. Here, we describe in detail the main methods of encapsulation of probiotics, including emulsion, extrusion, and spray-drying techniques.

Key words Probiotic, Encapsulation, Emulsion, Extrusion, Spray-drying

1 Introduction

The consumption of probiotic products has increased exponentially due to the range of benefits these microorganisms can offer to human health. However, it is still a challenge to ensure the viability of probiotics to the consumer, as they have a noticeable loss of viability after passing through the digestive tract. In addition, when incorporated into commercial products, intrinsic or processing factors such as low pH, high water activity, or high cooking temperatures can negatively affect the viability of microorganisms [1].

Microencapsulation emerges as an alternative to circumvent these limitations. This technique is based on trapping probiotics within an encapsulating matrix, ensuring safe delivery to the intestine at appropriate therapeutic levels to provide human health benefits [2]. Several microencapsulation techniques can be used to encapsulate probiotics (Table 1). However, emulsion, extrusion, and spray-drying techniques occupy a prominent place, considering

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Table 1
Encapsulation techniques used to microencapsulate probiotics

Encapsulation technique	Probiotic strain	Wall materials	Encapsulation yield (%)	References
Emulsion	*Lactiplantibacillus plantarum* (MT, ZH593)	Alginate	27–82	[7]
Extrusion	*Limosilactobacillus reuteri* (DSM 20016)	Alginate and (tamarind gum or mutamba mucilage or cassia tora gum or psyllium mucilage or konjac gum)	93–97	[8]
Spray-drying	*Bifidobacterium animalis* subsp. *lactis* BB-12	Full-fat goat's milk and/or prebiotics (inulin and/or oligofructose)	94–97	[9]
Freeze-drying	*Lactobacillus acidophilus* (La-05), *Lacticaseibacillus casei* (Lc-01)	Microalgae *Spirulina platensis*, *Chlorella vulgaris*, *Scenedesmus quadricauda*, and *Lagerheimia longiseta*	80–92	[10]
Supercritical	*Bifidobacterium animalis subsp. lactis* BB-12, *Bifidobacterium longum* BB-46	Poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid)	Not shown	[11]
Liposome	*Lacticaseibacillus rhamnosus* (ATCC 10754)	Lecithin and (chitosan or gelatin)	81–87	[12]
Electrospinning	*Lacticaseibacillus rhamnosus* 1.0320	Pectin and poly (vinyl alcohol)	Not shown	[13]
Microfluidics	*Saccharomyces cerevisiae* (PDC1-GFP)	Alginate	Not shown	[14]
Layer-by-layer	*Ligilactobacillus salivarius* Li01 (Li01)	Chitosan and alginate	Not shown	[15]
Fluidized bed	*Lactobacillus acidophilus* (PTCC 1643)	Xanthan, alginate, chitosan, and gellan	35–78	[16]
3D printing	*Bifidobacterium lactis* (HOWARU® Bifidous) *Lactobacillus acidophilus* (HOWARU® Dophilus)	Alginate and gelatin	Not shown	[17]

their low cost, simplicity of handling, and the possibility of producing large-scale microcapsules. Thus, throughout this chapter, we will address only these most used techniques.

The encapsulating matrix can be formed using different wall materials, also known as carriers. Sodium alginate has been widely used due to its low cost, biocompatibility, food grade, and targeted delivery of probiotics (soluble in basic medium, for example, in the intestine) [3]. Wall materials such as chitosan, gelatin, milk proteins, pectin, carrageenan, prebiotics, and different types of starch have also occupied a prominent place for the microencapsulation of probiotic strains. The criteria for choosing a suitable encapsulating agent are mainly based on its physicochemical properties (molecular mass, solubility, glass transition temperatures, crystallinity, film formation, and emulsifying properties). A good wall material must also be easy to handle during the encapsulation process. In addition, it cannot react or injure the probiotic strain during the encapsulation and storage process and, finally, it must meet the solubility properties of the microcapsule by releasing the probiotics at the site of action [1]. To describe the methodology of this chapter, we will consider alginate (ALG) and whey proteins (WPI) as encapsulating agents and the strain of *Lacticaseibacillus rhamnosus* GG as active material. Alginate was chosen because it is necessary to use a hydrocolloid for the crosslinking process in the emulsion and extrusion methods. In addition, it is considered GRAS (Generally Recognized as Safe) and low cost. However, other wall materials have been widely used [4–6].

2 Material

For the production of microcapsules, the following materials are needed:

- Freeze-dried probiotic cells;
- De Man Rogosa and Sharpe (MRS) broth;
- Glycerol.
- Bacteriological oven;
- Centrifuge;
- Saline solution;
- Soybean oil;
- Alginate (ALG);
- Whey proteins (WPI);
- Calcium carbonate;

- Acid organic;
- Span 80;
- Calcium chloride;
- Spray-drier.

3 Methods

3.1 Preparation of Probiotic Suspension

To obtain the stock solution, freeze-dried probiotic cells can be rehydrated in sterile skim milk (25 g L^{-1}) or with De Man Rogosa and Sharpe (MRS) broth added with glycerol (20 g L^{-1}) and stored in sterile Falcon vials at −20 ± 2 °C [18] (*see* **Note 1**). Then, the stock solution is added to sterile MRS broth and incubated (37 ± 1 °C for 48 h) to reach the stationary phase (*see* **Note 2**). After the incubation time, the probiotic cells are harvested by centrifugation (1000 × *g*) for 10 min at a temperature of 25 ± 1 °C and washed twice with saline solution (0.9 g 100 mL^{-1}). Cell pellets should be kept at 4 ± 1 °C until encapsulation procedure.

3.2 Encapsulation of Probiotics by Emulsion

The emulsion technique consists of mixing two immiscible phases, called the dispersed or discontinuous phase, and the oily or continuous phase [1]. In this method, ALG gelation can be performed internally or externally (Fig. 1). In internal gelation, the alginate is previously solubilized with calcium carbonate, and then an aliquot of organic acid is added to the mixture after emulsification to

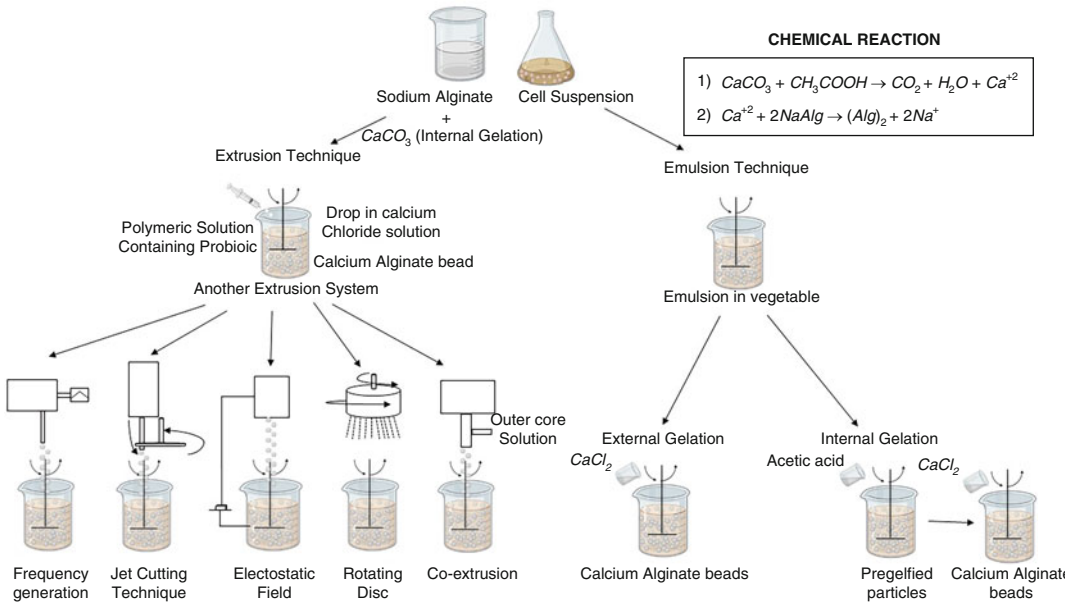

Fig. 1. Extrusion and emulsion technologies [19]. (Adapted from [19])

promote gelation. As the organic acid enters the aqueous phase, it interacts with calcium carbonate, releasing calcium ions and carbonic acid. The calcium ions react with the alginate through complexation with the carboxylic groups of the polymer, forming the "egg box model" structure [19]. On the other hand, in external gelation, the complexation reaction of the carboxylic groups of ALG occurs through contact with a solution of calcium chloride.

1. Preparing the dispersed phase: Mix 5% (w/v) of WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1% (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized.
2. Addition of cell biomass: Aseptically, an aliquot (~ 9 log CFU mL^{-1}) of the probiotic biomass should be added to the dispersed phase and then homogenized at 400 rpm for 5 min. It is recommended to add a biomass content that reaches a viable cell count of around 9 to 10 log CFU g^{-1} (*see* **Note 4**).
3. Preparing the continuous phase: Add 300 mL of soybean oil to a beaker. Add 3% (v/v) of an emulsifying agent (Span 80) in the same container and leave it under stirring at 400 rpm until complete homogenization (*see* **Note 5**).
4. Mixing the two phases: In a beaker, mix the dispersed and continuous phases and leave under stirring at 400 rpm for 20 min or until the complete formation of the emulsion (*see* **Note 6**).
5. ALG cross-linking process: While stirring, add an aliquot of a 1.5% (w/v) calcium chloride solution to form the gelled microcapsules (*see* **Note 7**). Then, turn off the agitation and add 200 mL of sterile distilled water to attract the microcapsules to the aqueous phase.
6. Collecting the microcapsules: Discard the emulsion supernatant, collect the microcapsules by vacuum filtration, and keep them at 4 °C until drying.
7. Drying of microcapsules: Gelled microcapsules can be dried in a spray-dryer, freeze-dryer, or fluidized bed dryer (*see* **Note 8**). After drying, the microcapsules can be packed in airtight packaging and kept at room temperature until use (*see* **Note 9**).

3.3 Encapsulation of Probiotics by Extrusion

The extrusion technique (Fig. 1) involves mixing the cellular biomass of the probiotic with the polymeric solution (ALG and WPI) and then forming droplets by passing the solution through a nozzle or atomizing nozzle [20].

1. Preparing the dispersed phase: Mix 5% (w/v) f WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1% (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized.

2. Addition of cell biomass: Aseptically, an aliquot (~ 9 log CFU mL^{-1}) of the probiotic biomass should be added to the dispersed phase and then homogenized at 400 rpm for 5 min. It is recommended to add an aliquot with a viable cell count of around 8 to 9 log CFU g^{-1} (*see* **Note 4**). It is worth emphasizing that the dispersed phase containing the hydrocolloid must be prepared just before use.
3. Forming the gelled microcapsules: Once the feed solution (FS) (polymer solution + probiotic) is prepared, the FS is dripped into a 1.5% (w/v) calcium chloride (*see* **Note 7**) gelling solution under stirring at 200 rpm. The dripping of the FS into the gelling solution is carried out using an atomizing nozzle. In this case, the FS is pumped by a peristaltic pump, and the droplets are quickly transformed into solid particles through the complexation of ALG with calcium ions. Another simplified form can be used, for example, using a syringe to perform the drip (*see* **Note 10**). After the dripping step, it is interesting to leave the microcapsules to rest (~20 to 30 min) in the $CaCl_2$ solution to solidify the microcapsules completely. The formation of large particles and the low production rate are the main disadvantages of this technique for use in the food industry. However, to overcome this, the extrusion process can be combined with ultrasound, jet cutting, electrostatic field, and rotating disk (Fig. 1).
4. Collecting the gelled microcapsules: Collect the microcapsules by vacuum filtration and keep them at 4 °C until drying.
5. Drying of microcapsules: Gelled microcapsules can be dried in a spray-dryer, freeze-dryer, or fluidized bed dryer (*see* **Note 8**). After drying, the microcapsules can be packed in airtight packaging and kept at room temperature until use (*see* **Note 9**).

3.4 Encapsulation of Probiotics by Spray-Drying

The spray-drying encapsulation technique (Fig. 2) is well established for large-scale industrial applications and is considered an economically viable technique. In this technique, the suspension containing the wall materials and probiotics is atomized in a drying chamber with concurrent hot air, which instantly removes water from the atomized solution [21]. Microcapsules are removed from the drying chamber by a negative pressure cyclone system and can be collected on the drying chamber bottom or in the collection flask (*see* **Note 11**).

1. Preparing the feed solution: Mix 5% (w/v) of WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1 (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized (*see* **Note 12**).
2. Addition of cell biomass: Aseptically, an aliquot of the probiotic biomass should be added to the dispersed phase and then

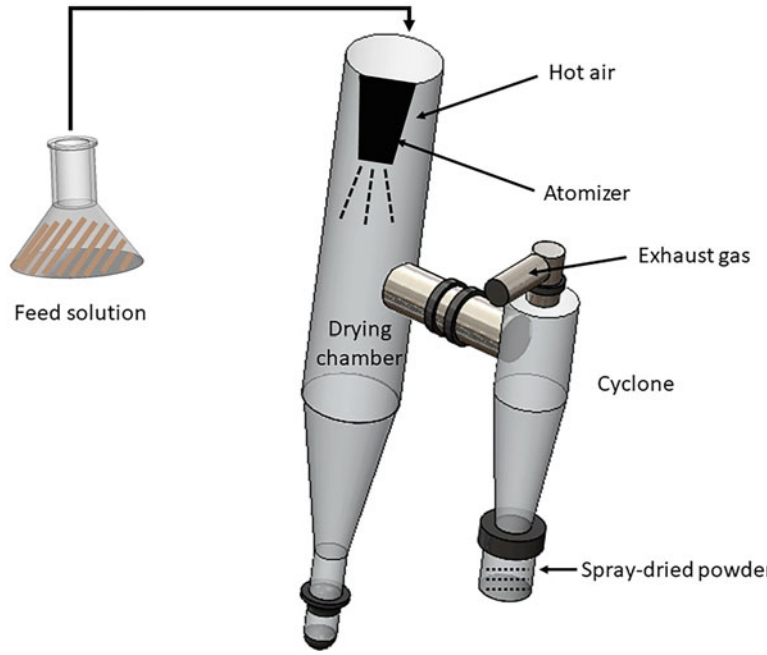

Fig. 2 Spray drying process schematic diagram [22]. (Adapted from [22])

homogenized at 400 rpm for 5 min. It is recommended to add an aliquot with a viable cell count of around 8 to 9 log CFU g^{-1} (*see* **Note 4**).

3. Encapsulation process: Turn on the spray-dryer equipment and operate it with the concurrent flow (*see* **Note 13**) with an inlet temperature of 150 °C and an outlet temperature of 50 °C (*see* **Note 14**). Program the drying airflow of 35 m^3 h^{-1}, and compressor air pressure of 0.7 MPa [23].
4. Then, turn on the peristaltic pump to pump the FS and program supply flow to 20 mL min^{-1}. It was found that slow drying kinetics leads to significant inactivation of the dehydration of *Lactiplantibacillus plantarum*, while a rapid drying rate could instantly stabilize the cells and thereby prevent this inactivation [24]. In addition, a high drying rate during the first stage of drying, when facilitated by hydraulic membrane permeability, may limit bacterial adaptation because of exposure for too short a time to the gradual withdrawal of moisture. It is recommended that the FS be kept under magnetic stirring at room temperature during the encapsulation process (*see* **Note 15**).
5. Before FS entry, sterile distilled water at room temperature must be pumping until stabilization of the inlet temperature.

6. Collecting the dry microcapsules: After complete evaporation of the water, collect the microcapsules from the collector located at the bottom of the equipment, store them in hermetic packages and keep them at room temperature until use (*see* **Note 9**).

The analysis of microcapsules is an important step in the microencapsulation process. Microcapsules must be characterized before use to observe their physical, chemical, and biological properties. Table 2 shows the characteristics of the probiotic microcapsules obtained by the emulsion, extrusion, and spray-drying techniques and the main characterization analyses.

4 Notes

1. You can use other cryoprotectants. MRS for LAB only, if strains from other species (*E. coli*, *Bacillus*, *Saccharomyces*), other broths should be used.
2. Cells in the stationary phase are more resistant and have a higher encapsulation yield than cells in the log phase [33].
3. Alginate should be added gently to not form lumps. You can place it on a foil film and spray it on the solution. Another way to avoid the formation of lumps is to homogenize them in warm water (40–50 °C).
4. Adding an aliquot of *L. rhamnosus* GG with a low viable cell count may compromise delivery to the gut at levels suitable for promoting human health.
5. Any oil can be used. The emulsifying agent is chosen according to the lipophilic hydrophilic balance (LHB); generally, the most used are Tween 80 and Span 80.
6. Using slow agitation rates (400–500 rpm) is recommended. High agitation rates can damage the probiotics'cell wall.
7. Other types of salt can be used for ALG crosslinking, such as calcium citrate. However, it is desirable to use low concentrations. High salt concentrations have a detergent effect, which dissolves bacterial membranes and even causes cell death.
8. Another drying method can be used. However, those are more commonly used. The drying of microcapsules is important both from a microbiological and technological point of view, as it increases the lifespan of microorganisms. In addition, drying the microcapsules makes it possible to incorporate probiotics into low-moisture food matrices.
9. These microcapsules can be used in the products described in other chapters of this edition to improve survivability in processing, storage, and TGI.

Table 2
Characteristics of the probiotic microcapsules obtained by the emulsion, extrusion, and spray-drying techniques and the main characterization analyses

Characteristics of the probiotic microcapsules	How to determine?	Why perform this analysis?	Ideal characteristics	Reference
Size and morphology	**Size:** Laser diffraction method using Mastersizer or dynamic light scattering (DLS) LUMiSizer	The size and morphology of the microcapsules are influenced by the wall materials and the encapsulation technique employed	The microcapsules should not exceed 100 μ in size	[25]
	Morphology: Optical microscopy, fluorescence microscopy, and scanning electron microscopy (SEM)	Microcapsules' size can affect their performance in protection, probiotic delivery, and the sensory quality of the food product incorporated with the microcapsules	They may have a regular shape (e.g., spherical, tubular, and oval) or an irregular shape	
Chemical structure and surface chemistry	Fourier transform infrared spectroscopy (FTIR)	FTIR identifies the functional groups after chemical modification, provides insights into the interaction between microcapsule components, confirms the cross-linking, and probes the degradation of the polymeric matrix	Not applicable	[25]
	Raman spectroscopy	Confirms immobilization and encapsulation of probiotic		
Density: Bulk density and true density	Multi-volume pycnometer and burette containing toluene	The density of microcapsules is a significant factor in the processing, storage, packaging, transportation, and commercialization of the product	Not applicable	[25, 26]

(continued)

Table 2
(continued)

Characteristics of the probiotic microcapsules	How to determine?	Why perform this analysis?	Ideal characteristics	Reference
Porosity	The porosity of the microcapsules can be calculated via the relationship between the bulk and true density of the sample SEM and Brunauer-Emmett-teller (BET) techniques	Porosity is defined as the void fraction in the powder sample. It is an important property that plays an important role in the stability of probiotic powders	Low porosity is desirable, as the presence of pores in the microcapsules can favor the permeation of substances harmful to probiotics, for example, stomach acid	[25, 27]
Water activity (a_w), moisture content, and hygroscopicity	**a_w:** Water activity analyzer (e.g., aqua lab, decagon devices) **Moisture content:** Gravimetrically with heat **Hygroscopicity:** Calculated as the weight of the water absorbed per mass of the sample (%), and can be determined by exposing the microbeads to a NaCl saturated solution for a period of time	Influences the stability of encapsulated probiotics during storage	A high a_w, moisture, and hygroscopicity imply a faster decline in viability during storage a_w around 0.3 is considered satisfactory for dried probiotic microcapsules High hygroscopicity tends to form clusters of microcapsules	[28, 29]
Thermal analysis	Differential scanning calorimetry (DSC)	Thermal stability of wall materials	Not applicable	[30]

Type of order present in powders: Amorphous or crystalline	X-ray diffraction	The crystals could damage the cells, which would reduce the viability of microorganisms, making the amorphous structure interesting Amorphous solids are in general more soluble, and the crystallization may entail a negative impact on the handling properties	Amorphous solids	[31]
Encapsulation process	**Encapsulation yield (EY):** Measured based on the ratio of the number of viable entrapped bacteria to the number of free bacteria	Determines the effectiveness of entrapment within microcapsules and survival of viable cells during the microencapsulation procedure	Microencapsulation can be considered successful when it yields relatively higher EY	[25]
Simulated gastrointestinal conditions	Infogest 2.0	Determines the stability of encapsulated probiotics in simulated gastrointestinal conditions such as mouth, stomach, and intestine	Probiotics should reach the intestine in adequate doses to promote human health	[32]
Thermal stability assay	Bath with controlled temperature	Determines the thermal stability of encapsulated probiotics	Probiotics should be resistant to elevated temperatures Heat-resistant encapsulated probiotics favor the probiotication of thermo-processed foods	[9, 30]
Storage stability	BOD incubator oven	Determines the stability of encapsulated probiotics during storage at different temperatures	It is desirable that probiotics remain stable for long periods	[30]

10. The formation of large particles and the slow production rate are the main disadvantages of this technique for use in the food industry.
11. For probiotic microcapsules, the ideal in bench spray-dryers or pilots is to collect only the product from the collector due to the greater control of the exit temperature of the process.
12. During the process, encapsulated microorganisms can undergo several stresses, including heat stress and dehydration. Encapsulating agents such as gelatin, gum arabic, and cellulose acetate phthalate has been reported as protective agents capable of forming a physical barrier resistant to hot air [21]. In addition, disaccharides are encouraged as they can preserve the structure of probiotic cell proteins and membranes through a connection at sites that previously interacted with water [34].
13. Spray flow can be applied in three ways (concurrent, countercurrent, or mixed flow). However, the choice of spray flow will depend on the direction in which air and liquid (e.g., feed solution) enter the drying chamber. In the first case (concurrent), the product is in contact with the colder air, preferable for drying thermosensitive materials, such as probiotics.
14. The lower the T_{outlet}, the higher the post-drying viability. T_{outlet} is therefore considered to be the principal drying parameter that affects the viability of spray-dried LAB, and any lack of monitoring and control of the latter may be markedly detrimental [21].
15. Agitation prevents materials in solution from settling.

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