

Review

Probiotic encapsulation

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Encapsulation technologies are used to keep probiotic cell viable throughout storage, commercialization and use in food products, so that these cells are active during their passage through the gastrointestinal tract. This review describes the most common encapsulation technologies and encapsulation materials used for maintaining the viability of probiotic bacteria under adverse external conditions. Illustrations are presented to facilitate the understanding of the various encapsulation methodologies. Supercritical fluid technologies for encapsulation as well as new wall materials are discussed. It is concluded that several variables affect the viability of encapsulated probiotic cells and therefore optimization tools including response, genetic algorithms, quadratic sequential programming are needed for appropriate material selection.

Key words: Lactic acid bacteria, lyophilization, spray drying, extrusion, cellular viability.

INTRODUCTION

Probiotics, from the Greek word meaning "for life", are defined as living organisms that provide health benefits to the host when ingested in sufficient quantities (Quigley, 2010). Lactic acid bacteria (LAB) are the most commonly used probiotic microorganisms due to their beneficial effects on the gastrointestinal tract. Probiotic bacteria are used in the food industry due to various beneficial properties including reduction of irritable bowel syndrome symptoms after *Bifidobacterium infantis* 35624 intake, immunomodulatory effects, and cholesterol reduction (FAO/WHO, 2006).

The administration of *Lactobacillus rhamnosus* LGG during acute rotavirus diarrhea in children, decreased the diarrhea duration vs. placebo (Jankovic et al, 2010); however, the use of other LAB (e.g., *Lactobacillus johnsonii*) was associated with gastritis by *Helicobacter pylori* (Pantoflickova et al., 2003).

Antibiotics are used in cattle to treat bacterial infections

and to promote growth when used in low concentrations. However, the widespread use of antibiotics caused resistance to pathogenic bacteria. Probiotic organisms are good alternatives to the widespread use of antibiotics (Edens, 2003). The gastrointestinal tract of calves is sterile at birth, and organisms are introduced from vaginal and fecal microbiota, and from the environment (Rosmini et al., 2004).

In all cases, probiotic bacteria should remain alive from the time they are consumed until their settlement in the intestine. This is difficult since the bacteria must bypass extreme acidic pH in the gastrointestinal tract. Encapsulation of probiotic bacteria is an alternative that provides protection for living cells exposed to an adverse environment (Burgain et al., 2011). It also helps food materials to resist processing and packaging conditions, improving taste, aroma, stability, nutritional value and product appearance (Parra-Huertas, 2010).

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Probiotic encapsulation of LAB has been proposed as an efficient technology to improve viability and preserve metabolic activity in the gastrointestinal tract (Picot and Lacroix, 2004), and to ensure viability during long-term storage (Zuidam and Nedovic, 2010). Viability is defined as the number of trapped (encapsulated) probiotic cells (cfu g^{-1}) that remain viable in their site of action to produce a beneficial health effect to the host (Krasaekoopt et al., 2003; De Vos et al., 2010). Encapsulation has been successfully used to improve cell viability during storage of several LAB including *Lactobacillus paracasei* NFBC 338 by spray-drying (Desmond et al., 2002), *Lactobacillus casei* NCDC-298 by emulsification (Mandal, et al., 2006) and *L. casei* by extrusion (Sandoval-Castilla et al., 2010), among others.

This review on probiotic encapsulation discusses materials and techniques used for encapsulation, and factors that affect the viability and controlled release of cells. New potential research avenues are also briefly discussed.

ENCAPSULATION

Encapsulation is defined as a process that entrap a substance into another substance, producing particles in the nanometer (nanoencapsulation), micrometer (microencapsulation) or millimeter scale (Lakkis, 2007; Burgain et al., 2011).

The encapsulated substance is usually called core material, active agent, filler agent, internal phase, or payload phase. A substance used to encapsulate is called coating membrane, shell, carrier or wall material, external phase or matrix. For specific encapsulation processes such as freeze-drying, the substances used to encapsulate are also called cryoprotectants. The wall material used in food products or processes should be food grade and must be able to form a barrier between the active agent and its surroundings (Zuidam and Nedovic, 2010).

Different types of encapsulates (reservoir, matrix and coated matrix) might be characterized (Figure 1). The reservoir type has a layer around the core material (also called capsule). The matrix type has the active agent dispersed over the carrier material and can be also found on the surface. A combination of these two types gives a third encapsulate called coated matrix, in which the active agent is a capsule covered by an additional layer (Lakkis, 2007).

Electron microscopy techniques are useful to obtain information on size range of bacteria-loaded and empty microcapsules, matrix microstructure and any matrix changes caused by the entrapped bacteria. By using cold stage scanning electron microscopy (cryo-SEM), it is possible to study the structure, configuration, and size distribution of capsules, and also differentiate capsules with or without bacteria. Cryo-SEM, including freeze-

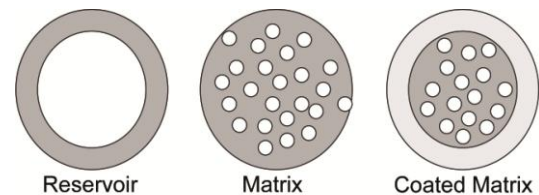


Figure 1. Types of encapsulates (Adapted from Zuidam and Nedovic, 2010).

fracture, allows observing details of the matrix and the interaction between the carrier material and the bacteria. It is also possible to observe empty spaces around the bacteria. Transmission electron microscope (TEM) could be used to study the matrix microstructure, detect subtle changes both in bacteria and in the matrix, and also provide a more detailed view of the differences in the porosity of capsules with or without bacteria (Allan-Wojtas et al., 2008).

MATERIALS FOR ENCAPSULATION

Carrier materials should serve as protection for probiotics and also be safe for consumption, that is, Generally Recognized As Safe (GRAS) and cost effective, since a high cost will directly influence the value of the final product. Low cost carrier materials include starches, inulin, pectin and most carbohydrates (De Vos et al., 2010). Other materials such as alginate and trehalose are often used but at a higher cost. Main materials for encapsulation are discussed below.

Polysaccharides

Agar, sodium alginate, carrageenan, gum arabic, chitosan, dextrans, starch and cellulose (ethyl-cellulose, acetyl-cellulose, methyl-cellulose, carboxymethyl-cellulose, nitrocellulose) are the principal carrier materials used for encapsulation. Sodium alginate is the most commonly used material, compatible with almost all encapsulation methods, and usually used in combination with other components (Burgain et al., 2011). It is a linear, unbranched amorphous copolymer composed of β -D-manuronic acid (M) and α -L-guluronic acid (G) linked by 1 \rightarrow 4 bonds. The M and G units on alginates can be randomly arranged or organized as heterogeneous or homogeneous sequences. The chemical composition and distribution of a sodium alginate sequence depends on the species and parts of algae used in extraction (Fu et al., 2011).

Sodium alginate is widely used as a gelling agent, due to its ability to form hydrogels with divalent cations, such

as Ca^{+2} , Ba^{+2} o Sr^{+2} under moderate conditions. The hydrogel is formed because the blocks of guluronic acid bond with cations, resulting in a three-dimensional network of alginate filaments that are held together with ionic interactions. The model that best describes this network is the "egg-box model" (Simpson et al., 2004). Sodium alginate at a concentration of 20 g/L in combination with milk whey protein at 10 g/L has been used to encapsulate *L. plantarum* 299v, *L. plantarum* 800 and *L. plantarum* CIP A159 by freeze-drying (lyophilization).

Results indicate that encapsulated strains incubated in the gastric juice have greater viability than free bacteria and are able to survive the intestinal environment (Gbassi et al., 2010). Using chitosan at a concentration of 1% w/v to encapsulate extruded strains of *Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994, and *L. casei* 01, resulted in greater viability for *L. acidophilus* and best protection *L. casei* cells. No carrier material was able to keep *B. bifidum* viability because of its low resistance to acidic pH (Krasaekoopt, et al., 2004).

A mixture of alginate and modified starch are used to encapsulate the probiotics *L. acidophilus* and *B. lactis* by emulsion to incorporate them into yogurt. Encapsulated probiotics showed greater viability in storage as compared to free cells. A sensory analysis found that the use of encapsulated cells in yogurt did not alter color, acidity or flavor properties (Kailasapathy, 2006).

Cellulose acetate phthalate is used as a carrier material to prepare microcapsules with *L. acidophilus* (La-05) and *B. lactis* (Bb-12) by spray drying. Studying the resistance of microorganisms at dry temperature and tolerance to a simulated environment with pH and bile salts that are similar to those in the human stomach and intestine, Favaro-Trindade and Grosso (2002) found no reduction in the viability of *B. lactis* with an inlet temperature of 130°C and outlet of 75°C in the spray dryer. For *L. acidophilus*, a two log cycle reduction in viability was observed. After 2 h of incubation in solutions with pH 1 and 2, the microcapsules were effective in protecting the microorganisms and similar results were obtained with bile salt solutions (Favaro-Trindade and Grosso, 2002).

Oligosaccharides

Corn syrup, sucrose and maltodextrin are commonly used. When an appropriate mix of maltodextrin and trehalose was used as carrier media to encapsulate *L. paracasei* by spray cooling or freezing, high bacterial viability was obtained. The concentration of trehalose helped increase cell viability due to its high osmotic pressure (Semyonov et al., 2010).

Lipids

Waxes, paraffin, diglycerides, monoglycerides, fats, stearic

acid, triestearins and oils are mainly used. When sesame oil was used to encapsulate *Lactobacillus delbrueckii subsp. bulgaricus* by emulsion, 5.4% increase in cells viability was observed under refrigeration conditions vs. free cells. Better cell viability of the encapsulated bacteria under simulated intestinal conditions was also observed (Hou et al., 2003).

Proteins

Gluten, casein, whey protein, albumin, are commonly used protein-based encapsulation materials. Picot and Lacroix (2004) worked with milk whey protein as a carrier material to encapsulate *Bifidobacterium breve* R070 and *Bifidobacterium longum* R023 by spray drying to be later included into yogurt. The results indicated that whey protein can increase the tolerance of the bacteria to acidic pH, therefore whey proteins have a great potential to be used in probiotics and in products where bacteria need to be viable when reaching the gastrointestinal tract (Picot and Lacroix, 2004). Also, when whey proteins were used to encapsulate *L. rhamnosus* by extrusion in a simulated gastrointestinal environment, the protein capsules formed a matrix providing protection to *L. rhamnosus* in acidic pH and produced an efficient controlled release of biomolecules with a subsequent absorption *in situ* at the specific destination (Doherty et al., 2011). Encapsulation materials and methods used to encapsulate LAB are presented in Table 1.

Encapsulation techniques: Operating parameters, advantages and disadvantages

Microencapsulation technologies were developed and applied successfully to protect probiotic bacteria (Table 1) from damage caused by external factors such as drying, packaging and storage conditions (e.g., time, temperature, moisture and oxygen), and the degradation in the gastrointestinal tract, especially due to extreme pH (2.5 to 3.5) of gastric juices and bile salts (Kailasapathy, 2006). The selection of the encapsulation method depends on the required particle average size, the physical and chemical properties of the carrier material, the applications of the encapsulated material, the required release mechanism and cost. These parameters need to be studied for each specific organism and process (Parra-Huertas, 2010; Burgain et al., 2011).

Encapsulation methods

Spray drying

Spray drying is an appropriate technique for industrial applications on a large scale. A liquid mixture is atomized

Table 1. Encapsulation materials and methods used to encapsulate lactic acid bacteria (LAB).

Lactic acid bacteria	Encapsulation technique	Encapsulation material	Reference
<i>Lactobacillus rhamnosus</i> GG	Extrusion	Whey protein	Doherty et al., 2011.
<i>Lactobacillus acidophilus</i> 547, <i>Bifidobacterium bifidum</i> ATCC 1994 y <i>Lactobacillus casei</i> 01, <i>Lactobacillus acidophilus Bifidobacterium lactis</i> (Bb-12)	Extrusion	Chitosan, poly-L-lysine, sodium alginate.	Krasaekoopt et al., 2004
<i>Bifidobacterium breve</i> R070 y <i>Bifidobacterium longum</i> R023	Spray drying	Cellulose acetate phthalate	Favaro-Trindade and Grosso, 2002
<i>Lactobacillus paracasei</i>	Spray drying	Whey protein	Picot and Lacroix, 2004.
<i>L. plantarum</i> 299v, <i>L. plantarum</i> 800 y <i>L. plantarum</i> CIP A159	Spray freeze-drying	Maltodextrin and trehalose	Semyonov et al., 2010
<i>Lactobacillus acidophilus</i> y <i>Bifidobacterium lactis</i>	Lyophilization	Sodium alginate - whey protein	Gbassi et al., 2010
<i>Lactobacillus delbrueckii ssp. bulgaricus</i> .	Emulsion	Sodium alginate and modified starch	Kailasapathy, 2006
<i>Lactobacillus casei</i> NCDC-298	Emulsion	Sesame oil	Hou et al., 2003
<i>L. rhamnosus</i> GG y <i>L. acidophilus</i> NCFM	Emulsion	Sodium alginate – soybean oil	Mandal et al., 2006.
<i>Lactobacillus paracasei</i> NFBC	Countercurrent spray	Sodium alginate - chitosan	Sohail et al., 2011
<i>Bifidobacterium</i> PL1	Spray drying	Acacia gum – reconstituted skim milk	Desmond et al., 2002
<i>Bifidobacterium infantis</i>	Spray drying	Starch	O’Riordan et al., 2001
<i>Lactobacillus bulgaricus</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus rhamnosus</i> , <i>Enterococcus durans</i> , <i>Enterococcus faecalis</i>	Emulsion- Spray drying	Canola oil - sodium caseinate - fructooligosaccharide -dehydrated glucose syrup	Crittenden et al., 2006
<i>Carnobacterium divergens</i> , <i>Lactobacillus salivarius</i> y <i>Lactobacillus sakei</i>	Lyophilization	Skim milk – sorbitol - monosodium glutamate	Carvalho et al., 2003
<i>Lactobacillus reuteri</i> C10	Spray drying	Skim milk powder	Silva et al., 2002
<i>Lactobacillus F19 Bifidobacterium Bb12</i>	Lyophilization	Skim milk - sucrose-lactose	Khoramnia et al., 2011
<i>Lactobacillus F19 Bifidobacterium Bb12</i>	Emulsion - Lyophilization	Sodium caseinate – transglutaminase - sunflower oil	Heidebach et al., 2010

in a tank using a nozzle or disk and the solvent is evaporated after coming into contact with hot air or gas (Yañez et al., 2002) (Figure 2). Its application in *Lactobacillus* spp. (Desmond et al., 2002) and *Bifidobacterium* (O’Riordan et al., 2001) received considerable interest.

The process parameters to be considered include:

1. Air flow configuration: co-current or counter-current flow
2. Strain type and its pre-adaptation to the carrier material (Corcoran et al., 2004)
3. Carrier material: low viscosity for easy flow, tasteless flavor and high solubility (Vega and Roos, 2006)
4. Drying temperature: the number of viable probiotics linearly decreases with outlet air temperature. The output

optimum air temperature should be as low as possible and the intake air temperature must be in the range of 150 to 170°C. Slow feed rate allows temperatures close to 80°C

5. Drying time: a short drying time improves the probiotics viability

6. Storage conditions: the survival of probiotics is optimal with low water activity (<0.25) (Zuidam and Nedovic, 2010) and low temperature (4°C). These conditions were used in yogurt and are usually recommended for good stability of dried cultures throughout storage (Picot and Lacroix, 2004). Temperatures between 19 and 25°C (O’Riordan et al., 2001) were used to assess the viability of encapsulated bacteria maintained under adverse environmental conditions.

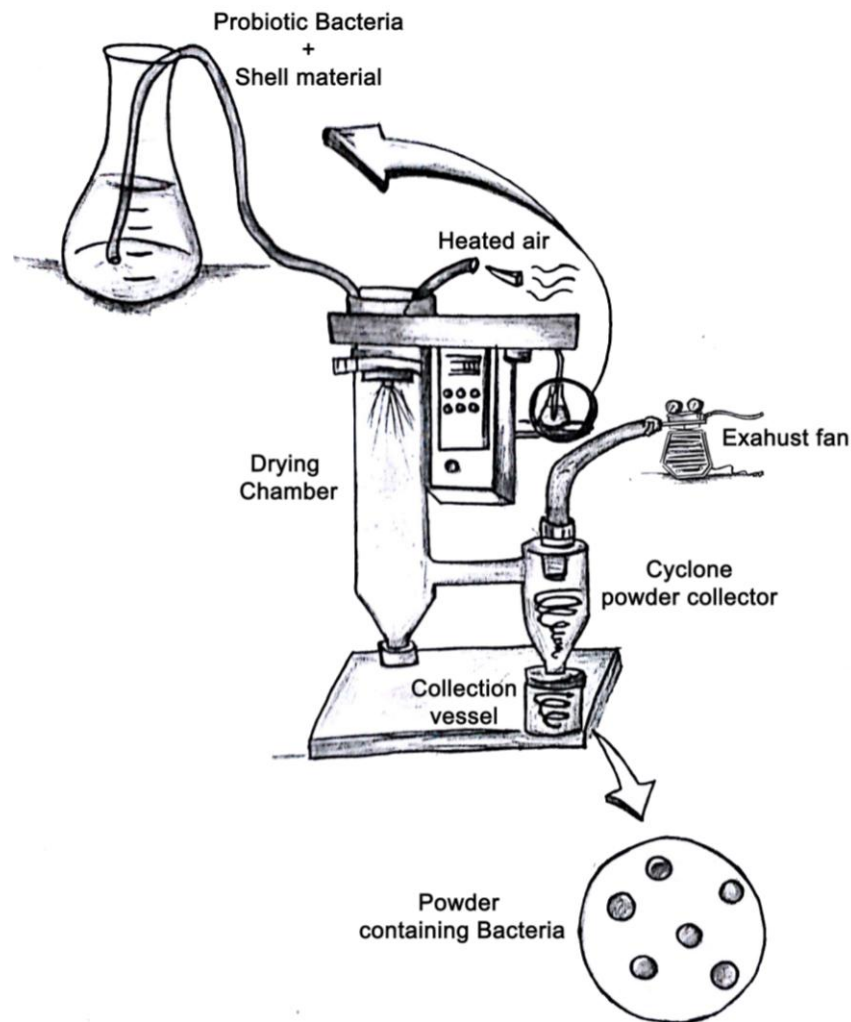


Figure 2. Schematic diagram of the spray-drying encapsulation method.

Spray drying has been used to encapsulate *L. paracasei* NFBC using gum arabic and reconstituted skim milk as carrier materials. Intake air at a constant temperature of 170°C was used. The culture was sprayed inside the drying chamber using a nozzle for two fluids, allowing the product to be dried almost instantly with very short residence time. Outlet temperature varied between 95 and 105°C. The capsules of *L. paracasei* NFBC prepared at temperatures between 95 and 105°C exhibited a 3 log increase in cell viability vs. free cells under the same conditions (Desmond et al., 2002).

Crittenden et al. (2006) obtained small capsules of *B. infantis* (15 to 20 µm in diameter) with a low water activity (0.2-0.3), using an oil in water emulsion prepared with canola oil, caseinate, fructooligosaccharide and dehydrated glucose syrup or starch resistant to microfluidization, as carrier materials. The inlet and outlet air temperature

were 160 and 65°C, respectively. Also, the viability of the bacteria at storage room conditions (25°C) and the ability of the carrier material to protect *B. infantis* in a simulated human stomach and small intestine environments were studied. Microencapsulation significantly protected the bacteria at room temperature and in a simulated stomach and small intestine conditions when compared with non encapsulated bacteria (Crittenden et al., 2006).

Lyophilization

Lyophilization is done by freezing the probiotic together with the carrier material (typically between -30 and -20°C), followed by vacuum sublimation of water at absolute pressure between 0.05 to 0.1 mBar and temperature between -50 to -30°C. Once lyophilized, cryoprotectants are added to preserve and stabilize the probiotic activity

during storage. The most common cryoprotectants are lactose, trehalose, sorbitol, sucrose, milk protein and skim milk (Semyonov et al., 2010).

Encapsulated probiotics by lyophilization have better storage stability, especially at low temperatures and inert atmosphere (nitrogen or vacuum) (Zuidam and Nedovic, 2010). Unfortunately, lyophilization is 4 to 7 times more expensive than spray drying (Chavez and Ledebor, 2007).

Carvalho et al. (2003) studied the effect of sorbitol and monosodium glutamate in solution with 11% skim milk on the storage viability of lyophilized *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Enterococcus durans*, *Enterococcus faecalis*. They reported a strong protective effect of sorbitol on the survival of the studied bacteria during storage. However, no significant differences in cell viability during lyophilization were observed. Monosodium glutamate after lyophilization showed an increase on LAB survival during storage. Consequently, the effect of each protective agent on the viability of a specific strain during or after the lyophilization process should be determined in a case by case basis.

Khoramnia et al. (2011) used response surface methodology (RSM) with central composite designs (CCD), to study the effect of cryoprotectants (skim milk, sucrose, and lactose) on the survival rate of the probiotic strain *Lactobacillus reuteri* C10 during lyophilization and storage for direct application in poultry. *L. reuteri* C10 has been characterized as having several probiotic properties. The central points used in the design were 12.5% (w/v) skim milk, 8% (w/v) sucrose, and 12.5% (w/v) lactose. The results showed that the presence of different combinations of cryoprotectants reduced the loss of cell viability during lyophilization. The loss of viability ranged from 0.26 to 0.66 log cfu/mL, while without cryoprotectants, values of 1.65 log cfu/mL were observed. The optimal combination of cryoprotectants for the preservation and storage of *L. reuteri* C10 was obtained with 19.5% skim milk, 1% sucrose and 9% of lactose. The survival rate of lyophilized *L. reuteri* C10 using the best combination of cryoprotectants and stored at 4 and 30°C for 6 months, was 96.4 and 73.8%, respectively.

Jin et al. (1998) using a supplemented diet with *Lactobacillus* lyophilized cultures to feed broiler chickens found that the presence of *Lactobacillus* cultures significantly increased body weight and feed : gain ratio of broilers during 0 to 6 weeks. RSM was used by Huang et al. (2006) to improve the viability of *L. delbrueckii* subsp. *bulgaricus* LB14 using sucrose, glycerol, sorbitol and skim milk during lyophilization. The results showed that the RSM not only helped in finding the optimal protective agent concentrations to maximize bacteria viability, but also provided adequate information to assess main effects and interaction among protective agents on cell viability.

Extrusion

The oldest and most common technique to produce capsules with hydrocolloids (e.g., alginate and carrageenan) consists of preparing a hydrocolloid solution, adding microorganisms and forming droplets by extruding the suspension through a syringe needle (laboratory scale) or an extruder (pilot scale) to free-fall into a hardening solution (e.g., calcium chloride) (Figure 3).

The size and shape of the formed pearl depend on the diameter of the nozzle and the distance between the nozzle and the CaCl₂ solution. This method is simple and cost effective. It does not cause cell damage and results in high cell viability (Krasaekoopt et al., 2003). The technology does not use harmful solvents and can be done under both, aerobic and anaerobic conditions. The main disadvantage of this method is that it is difficult to use in large scale production due to the slow formation of microspheres.

The survival of the probiotic microorganisms *L. acidophilus* 547, *B. bifidum* ATCC 1994, and *L. casei* 01 microencapsulated in chitosan-coated alginate pearls was evaluated in yogurt made with UHT milk and conventional pasteurization during storage at 4°C for 4 weeks. Sodium alginate (20 gL⁻¹) and chitosan (4 gL⁻¹) were used to prepare the pearls. The results showed that the survival of the encapsulated probiotic bacteria was greater vs. free cells in approximately 1 log cycle. During storage, the number of probiotic bacteria, with the exception of *B. bifidum*, remained above 10⁷ cfu g⁻¹, minimum recommended to ensure a therapeutic effect. The *B. bifidum* count fell below 10⁷ cfu g⁻¹ after 2 weeks of storage. The UHT treatment in yogurt did not alter the probiotic bacteria viability when compared with conventional thermal treatment (Krasaekoopt et al., 2006).

Soto et al. (2011) studied the use of macro-capsules from *L. casei* DSPV 318 T, a probiotic inoculum from bovine origin, in two formulations: sodium alginate (10 g/L) and sodium alginate (5 g/L) + corn starch (5 g/L). These mixtures were dispersed in containers of 1 and 2 mL, frozen at -20°C, immersed in boiling water and then placed in a CaCl₂ (0.1 M) solution to promote polymerization and maintain the capsules shape and size. The capsules were stored at 18 and 4°C and their viability was recorded for 63 days. The results showed that the refrigerated capsules had greater viability vs. the capsules kept at room temperature. It was concluded that probiotics had a shelf life of at least 2 months and could be used as culture initiators in calves.

The balance of the intestinal ecosystem may be negatively altered by stress situations, the use of antibiotics or by feeding calves with milk substitutes instead of colostrum. These practices can cause morbidity and mortality of young calves and economic losses. The regular administration of a probiotic inoculum of bovine origin can

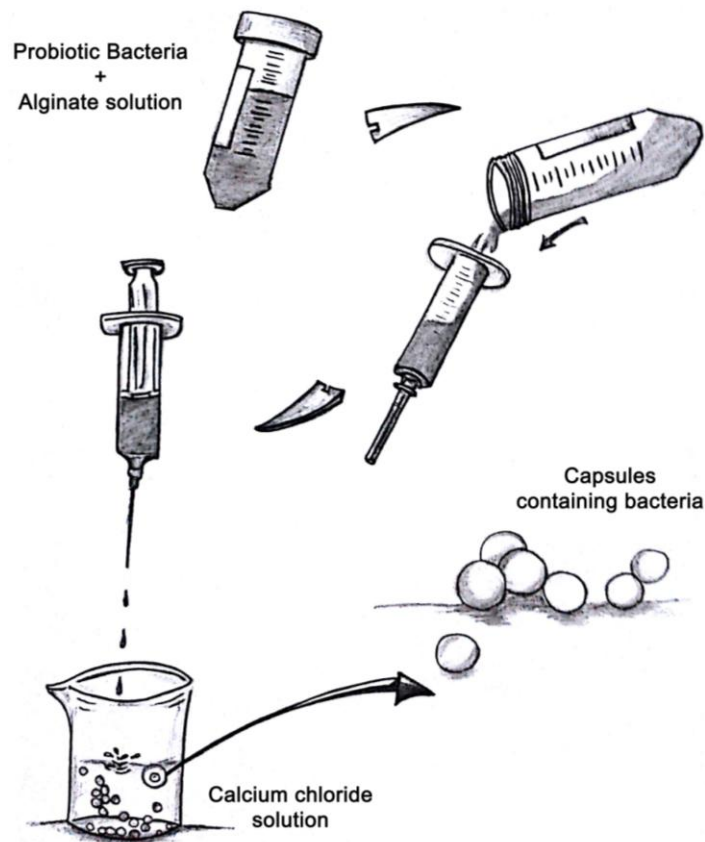


Figure 3. Schematic diagram of the extrusion encapsulation method.

promote a stable and balanced intestinal microbiota, and improve the calf's health (Soto et al., 2011).

Emulsion

This technique adds a small volume of a hydrocolloid suspension containing micro-organisms (discontinuous phase) to a large volume of vegetable oil (continuous phase). The mixture is homogenized to form water in oil emulsions by using an emulsifier. Once the emulsion is formed, it can be insolubilized to form gel capsules in the oil phase (Figure 4). The main disadvantage of this method is that it yields a wide range in particles size and shape (Burgain et al., 2011).

This technique has been used to encapsulate *L. casei* NCDC-298 in a matrix of sodium alginate, using soy oil as the continuous phase. 20 mL of alginate solution and 4 mL of cell suspension were mixed with 100 mL of soybean oil and 0.2% Tween 80, under continuous stirring. To hardened capsules and fragment the emulsion, 100 mL of 0.1 M calcium chloride were added. The results showed that encapsulation of *L. casei* NCDC-298 in sodium alginate, improved the viability of the bacteria in

simulated intestinal conditions and under thermal treatment (Mandal et al., 2006).

This emulsion technique is relatively new in the food industry and easy to scale up. It provides encapsulated and trapped core materials. The particle size formed by this method is smaller (25 μm - 2 mm) than the size produced by the extrusion method (2 to 5 mm). Pearl size formed by extrusion depends on size of the needle, while particle size produced by emulsification depends on speed of agitation and type of emulsifier. The need for vegetable oil in the formulation may increase operation costs when compared with the extrusion method (Krasaekoopt et al., 2003).

Spray cooling or freezing (spray freeze-drying)

This procedure combines common steps used in lyophilization (freeze-drying) and spray drying, and has the advantages of providing controlled size and higher specific surface area vs. spray-dried capsules. However, the method also has disadvantages including high-energy requirement, long processing time and 30-50 times higher cost vs. spray drying (Burgain et al., 2011).

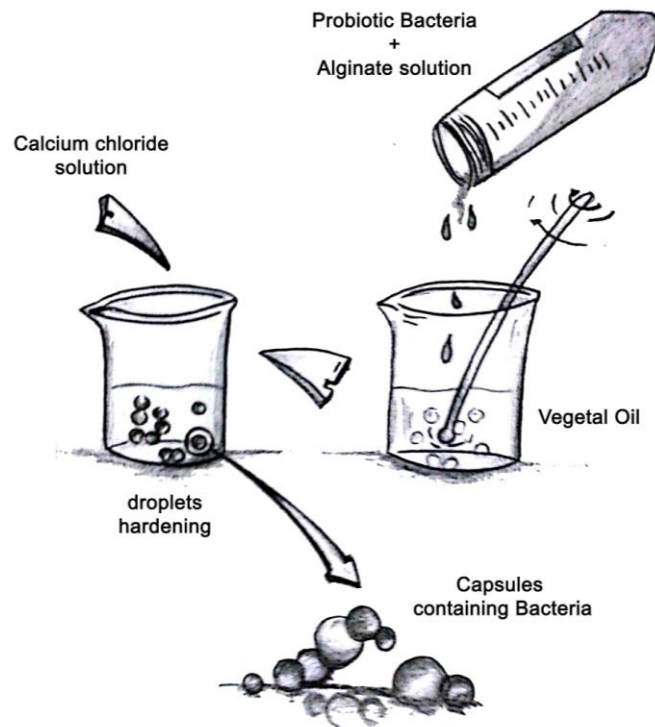


Figure 4. Schematic diagram of the emulsion encapsulation method (water-in-oil emulsion).

Table 2. Summary of encapsulation methods.

Encapsulation Method	Mechanism	Cost	Easy to Implement	Type of particle	Particle size (μm)
Spray drying	Dehydration	Low	Yes	Approximately spherical powders	3-100
Lyophilization	Sublimation drying	High	No	Irregular shapes	-
Extrusion	Reticulation	Low	Yes	Spherical capsules	1600-5000
Emulsion	Emulsification	Low	Yes	Spherical capsules	25-2000
Spray Freeze-Drying	Lyophilization-Spray drying	High	No	Approximately spherical powders	400-1400

Semyonov et al. (2011) encapsulated *L. paracasei* by spray freeze-drying using maltodextrin and trehalose as carrier materials. The technique consisted of spraying droplets containing a *L. paracasei* solution directly on liquid nitrogen and dehydrating the frozen droplets by lyophilization. Better bacteria viability was observed when using this technique than when using only lyophilization. Another technique is based on aerosols of alginate and CaCl_2 solutions flowing from opposite directions in a chamber (Sohail et al., 2011). The technique consists of mixing a liquid bacteria culture with 2% sodium alginate solution, and then pumping the solution in the upper part of a plexiglass cylinder and in 0.1 M CaCl_2 solution in the bottom. Alginate solution droplets come in contact with

CaCl_2 and fall to the bottom of the chamber from where the microcapsules are collected. Sohail et al. (2011) encapsulated *L. rhamnosus* GG and *L. acidophilus* NCFM using this technique and reported lower capsule sizes than those obtained by the traditional extrusion method. In addition, the micro-capsules were coated with chitosan, increasing the viability to simulated intestinal conditions. A summary of the encapsulation methods is presented in Table 2.

Use of enzymes in the encapsulation process

Transglutaminase enzymes have been used in probiotics encapsulation. The process is based on enzymatic gel

formation of the encapsulating material, and the subsequent application of one of the conventional encapsulation technologies. Heidebach et al. (2009) encapsulated a probiotic cell mixture with suspension of casein treated with transglutaminase and then applied the emulsion technique. This process provided 70 and 90% cell viability. The obtained capsules protected *Lactobacillus* F19 and *Bifidobacterium* Bb12 from pH damage levels simulating human stomach (Heidebach et al., 2009). These authors encapsulated *Lactobacillus* F19 and *Bifidobacterium* Bb12 with a water in oil emulsion and transglutaminase gel formation, and studied the effect of lyophilization and storage. Encapsulation was done using 15% (w/w) sodium caseinate as carrier material which was mixed with microorganisms and 10 international units of transglutaminase per gram of sodium caseinate at 40°C. Sunflower oil was finally added. The microcapsules were then centrifuged to separate the residual oil, lyophilized and stored at 4 and 25°C for 90 days. The results showed that the encapsulated *Bifidobacterium* Bb12 dropped ca. one log cycle in viability and the capsules showed an average of 3.8×10^9 cfu g⁻¹ when stored at 4°C for 90 days. In *Lactobacillus* F19, the reduction was almost 2 log cycles with approximately 1.7×10^8 cfu g⁻¹ at the end of storage time. The capsules contained the minimum required probiotic concentration (10^6 - 10^7 cfu g⁻¹) to have a therapeutic effect.

Stability of encapsulated materials

To determine the stability of encapsulated materials during storage, the glass transition temperature (T_g) measured needs to be established using differential scanning calorimetry (DSC) (Ndoye et al., 2007). The encapsulated product exhibits long shelf life when stored below glass transition temperature since deterioration due to bacterial proliferation and chemical reactions is very small. The low permeability of the carrier material at glass transition temperature is useful in preventing entrance of oxygen and preserving core materials. If storage temperature is set higher than glass transition temperature, various chemical reactions are accelerated because of the increase in the internal mobility of reagents and diffusion of oxygen (Qv et al., 2011).

Methods for controlling the release of encapsulated ingredients

Encapsulated probiotics need to be released from food products at the desired time and place. Understanding the chemistry of the carrier material allows controlling the release at a specific pH, temperature, and/or salt concentration. The release of the microcapsules must occur after crossing the gastrointestinal tract, releasing viable and

metabolically active bacteria (Picot and Lacroix, 2004). Water-insoluble microparticles can increase their tolerance to high acidic environments favoring the release of probiotic cultures to the gastrointestinal tract (Ding and Shah, 2007). Thermal, physical and the dissolution method are the most common release mechanisms.

In the thermal release mechanism, the encapsulating protector material melts at a certain temperature, usually releasing the ingredient during cooking. By altering the type of carrier material and its thickness, it is possible to assure the release of the ingredient within a few degrees of the target temperature. Physical release occurs by physical breaking of microcapsules. This mechanism is generally designed for ingredients that should be released during chewing. Factors to be considered are capsule size, strength and flexibility of coating. In the dissolution method, the majority of food products contain at least a small amount of water, which can be used to assure the release of an ingredient trapped in a water-soluble coating membrane (Lakkis, 2007).

RESEARCH IN ENCAPSULATION

The additional cost associated with probiotic encapsulation must be fully justified in terms of offering a clear performance improvement. Since cost is an important competitive factor, the search for low cost encapsulating materials that enable protection and proper micro-organism release is required.

Carrier materials that protect LAB and offer other benefits including functional, nutraceutical and prebiotic properties, should be studied (Crittenden et al., 2006).

In addition, there are natural compounds that provide specific functional properties, such as the *Aloe vera* gel, which has *in vivo* and *in vitro* immune modulatory activities (Reynolds and Dweck, 1999) of interest in the food industry (Martínez-Romero et al., 2006). This gel inhibits the growth of several pathogens, including *Staphylococcus aureus* and *Streptococcus agalactiae* (Reynolds and Dweck, 1999). It has been used for encapsulation by freeze-drying (Serna-Cock et al., 2012) with promising results as a material to encapsulate LAB, since it preserves the viability of *Weissella confusa* (83.3% survival rate) when compared with free cells. It also yielded higher viability (80%), when compared with cells encapsulated in a sodium caseinate (10%) solution.

A new encapsulation method based on the use of supercritical fluid technology has been proposed by Moolman et al. (2006). These authors used an inter-polymer complex formation in supercritical carbon dioxide. The method was used to encapsulate indomethacin and *Bifidobacterium longum* in a poly (vinyl pyrrolidone) – poly (vinyl acetate–co-crotonic acid) interpolymer complex. The encapsulation matrix was stable at low pH, but

was disintegrated at higher pH, triggering release of the encapsulated material. Interpolymer complex encapsulation showed potential for protection of probiotics and therefore for application in the food and pharmaceutical industries (Thantsha et al., 2009).

Aro et al. (2013) used oat polar lipids produced by supercritical fluid technologies in the encapsulation of probiotics. The protective effects of the oat polar lipids were evaluated by measuring the gas production, microbial activity, acetic and lactic acid production, and pH changes in different test mediums. The results demonstrated that the polar lipids from oats were able to suspend probiotic bacteria in such way that they stay viable after being put into frozen storage and then re-suspended in aqueous systems.

In vivo studies are necessary to understand cell viability and capsule stability in real environments. In a research conducted by Kanmani et al. (2011), *in vivo* test were performed using six fasting albino male Wistar rats. Each rat was fed orally with microcapsules made by extrusion containing the probiotic strain *Enterococcus faecium* MC13. After feeding, the rats were sacrificed at 2 h intervals until 12 h. The stomach and intestine of each rat were extracted and washed with sterile water to observe the microcapsules with a microscope. Results showed that up to 4 h, stability and uniformity in size were observed in the recovered microcapsules; after 6 h, capsules began to break and the carrier material (alginate) provided a direct and easy delivery of probiotic cells in the intestinal region.

Similarly, the minimum physical and chemical properties that make a material promising for encapsulation of biological compounds (probiotics) remain unknown. More research to set specific properties, such as viscosity, molecular weight, gel formation, composition and glass transition temperature, is needed for specific applications.

Conclusion

Given the number of variables involved in optimizing an encapsulation process for a bioactive molecule (for example, encapsulation material, encapsulation technique, release mechanism and processing conditions), tools including response surface, genetic algorithms and sequential quadratic programming should be used. The main stages in the optimization include diagnostic experiments that involve theoretically promising variables for the encapsulated material, proposing the encapsulation according to the experimental design, construction of response surface models, optimization model formulation improvement of optimization and verification of optimal conditions. Research focused on physical and chemical properties of encapsulation materials, *in vivo* tests for release mechanisms, encapsulation of low-cost materials that comply with probiotic and prebiotic functions, is

needed. It is essential to maintain stability, viability and cellular concentration during storage to ensure product efficiency for applications in food industry.

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