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Encapsulation Technology to Protect Probiotic Bacteria

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1. Introduction

Probiotic bacteria are used in production of functional foods and pharmaceutical products. They play an important role in promoting and maintaining human health. In order, to produce health benefits probiotic strains should be present in a viable form at a suitable level during the product is shelf life until consumption and maintain high viability throughout the gastrointestinal tract. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells [1]. Providing probiotic living cells with a physical barrier to resist adverse environmental conditions is therefore an approach currently receiving considerable interest [2].

The encapsulation techniques for protection of bacterial cells have resulted in greatly enhanced viability of these microorganisms in food products as well as in the gastrointestinal tract. Encapsulation is a process to entrap active agents within a carrier material and it is a useful tool to improve living cells into foods, to protect [3, 4, 5, 6, 7], to extend their storage life and to convert them into a powder form for convenient use [8, 9, 10, 11]. In addition, encapsulation can promote controlled release and optimize delivery to the site of action, thereby potentiating the efficacy of the respective probiotic strain. This process can also prevent these microorganisms from multiplying in food that would otherwise change their sensory characteristics. Otherwise, materials used for design of protective shell of encapsulates must be food-grade, biodegradable and able to form a barrier between the internal phase and its surroundings.

2. Probiotics

2.1. Definition

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer health benefits to the host [12], including inhibition of pathogenic growth,

maintenance of health promoting gut microflora, stimulation of immune system, relieving constipation, absorption of calcium, synthesis of vitamins and antimicrobial agents, and predigestion of proteins [13]. Several health benefits have been proved for specific probiotic bacteria, and recommendations for probiotic use to promote health have been published [14].

The term ''probiotic'' includes a large range of microorganisms, mainly bacteria but also yeasts. Because they can stay alive until the intestine and provide beneficial effects on the host health, lactic acid bacteria (LAB), non-lactic acid bacteria and yeasts can be considered as probiotics. LAB are the most important probiotic known to have beneficial effects on the human gastro-intestinal (GI) tract [15].

The effects of probiotics are strain-specific [16, 17, 18] and that is the reason why it is important to specify the genus and the species of probiotic bacteria when proclaiming health benefits. Each species covers various strains with varied benefits for health. The probiotic health benefits may be due to the production of acid and/or bacteriocins, competition with pathogens and an enhancement of the immune system [19]. Dose levels of probiotics depend on the considered strain [20], but 10^6–10^7 CFU/g of product per day is generally accepted [21].

2.2. Health benefits

There is evidence that probiotics have the potential to be beneficial for our health [22]. Multiple reports have described their health benefits on gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol, immune system stimulation, antimutagenic properties, anti-carcinogenic properties, anti-diarrheal properties, improvement in inflammatory bowel disease and suppression of Helicobacter pylori infection by addition of selected strains to food products [23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33].

The beneficial effects of probiotic microorganisms appear when they arrive in the intestinal medium, viable and in high enough number, after surviving the above mentioned harsh conditions [34]. The minimum number of probiotic cells (cfu/g) in the product at the moment of consumption that is necessary for the fruition of beneficial pharmaceutical (preventive or therapeutic) effects of probiotics has been suggested to be represented by the minimum of bio-value (MBV) index [35]. According to the International Dairy Federation (IDF) recommendation, this index should be ≥10^7 cfu/g up to the date of minimum durability [36]. Also, various recommendations have been presented by different researchers such as >10^6 cfu/g by all probiotics in yogurt [37, 38] and >10^7 cfu/g in the case of bifidobacteria [39]. Apart from the MBV index, daily intake (DI) of each food product is also determinable for their probiotic effectiveness. The minimum amount of the latter index has been recommended as approximately 10^9 viable cells per day [35, 38, 40].The type of culture media used for the enumeration of probiotic bacteria is also an important factor for determination of their viability, as the cell recovery rate of various media are different [35, 41].

Most existing probiotics have been isolated from the human gut microbiota. This microbiota plays an important role in human health, not only due to its participation in the digestion process, but also for the function it plays in the development of the gut and the immune system [42]. The mechanisms of action of probiotic bacteria are thought to result from modification of the composition of the endogenous intestinal microbiota and its metabolic activity, prevention of overgrowth and colonization of pathogens and stimulation of the immune system [43]. With regard to pathogen exclusion, probiotic bacteria can produce antibacterial substances (such as bacteriocins and hydrogen peroxide), acids (that reduce the pH of the intestine), block adhesion sites and be competitive for nutrients [44].

Recent studies have shown differences in the composition of the gut microbiota of healthy subjects [45], underlining the difficulties in defining the normal microbiota at microbial species level. Moreover, studies suggest that some specific changes in gut microbiota composition are associated with different diseases [46, 47]. This was confirmed by the comparison of the microbiome from healthy individuals with those of diseased individuals, allowing the identification of microbiota imbalance in human diseases such as inflammatory bowel disease or obesity [48, 49].

3. Encapsulation of probiotic living cells

Encapsulation is often mentioned as a way to protect bacteria against severe environmental factors [50, 51].The goal of encapsulation is to create a micro-environment in which the bacteria will survive during processing and storage and released at appropriate sites (e.g. small intestine) in the digestive tract. The benefits of encapsulation to protect probiotics against low gastric pH have been shown in numerous reports [50] and similarly for liquid-based products such as dairy products [21, 52].

Encapsulation refers to a physicochemical or mechanical process to entrap a substance in a material in order to produce particles with diameters of a few nanometres to a few millimetres. So, the capsules are small particles that contain an active agent or core material surrounded by a coating or shell. Encapsulation shell materials include a variety of polymers, carbohydrates, fats and waxes, depending of the core material to be protected, and this aspect will be discussed below in the this section.

The protection of bioactive compounds, as vitamins, antioxidants, proteins, and lipids may be achieved using several encapsulation technologies for the production of functional foods with enhanced functionality and stability. Encapsulation technologies can be used in many applications in food industry such as controlling oxidative reaction, masking flavours, colours and odours, providing sustained and controlled release, extending shelf life, etc. In the probiotic particular case, these need to be protected during the time from processing to consumption of a food product. The principal factors against them need to be protected are:

- Processing conditions (temperature, oxidation, shear, etc.)
- Desiccation (for dry food products)
- Storage conditions (packaging and environment: moisture, oxygen, temperature, etc.)

- Degradation in the gastrointestinal tract (low pH in stomach and bile salts in the small intestine).

Encapsulation technology is based on packaging of bioactive compounds in mili-, micro- or nano-scaled particles which isolate them and control their release upon applying specific conditions. The coating or shell of sealed capsules needs to be semipermeable, thin but strong to support the environmental conditions maintaining cells alive, but it can be designed to release the probiotic cell in a specific area of the human body. The scientific references related with probiotic encapsulation stress the degradation in the gastrointestinal tract, more than the processing conditions and the coating material usually employed can withstand acidic conditions in the stomach and bile salts form the pancreas after consumption. In this way, the protection of the biological integrity of probiotic bacteria is achieved during gastro-duodenal transit, achieving a high concentration of viable cells to the jejunum and the ileum.

The selection of the best encapsulation technology for probiotics needs to consider numerous aspects in order to guarantee the survival of bacteria during the encapsulation process, in storage conditions and consumption, as well as the controlled release in the specific desired area of gut. So, there are two important problematic issues considering probiotic encapsulation: the size of probiotics which exclude the nanoencapsulation technologies and the difficulties to keep them alive.

In this section the most common techniques used for microencapsulation of probiotics will be presented (Sect. 3.1), as well as the most usual microcapsule coating or shell materials (Sect. 3.2) and some marketing considerations for their application in food products (Sect. 3.3).

3.1. Main techniques for microencapsulation of probiotics

3.1.1. Spray-drying

Spray-drying is a commonly used technique for food ingredients production because it is a well-established technique suitable for large-scale, industrial applications. The first spray dryer was constructed in 1878 and, thus, it is a relatively old technique compared with competing technologies [53]. This technique is probably the most economic and effective drying method in industry, used for the first time to encapsulate a flavour in the 1930s. However, it is not so useful for the industrial production of encapsulated probiotics for food use, because of low survival rate during drying of the bacteria and low stability upon storage.

Drying is an encapsulation technique which is used when the active ingredient is dissolve in the encapsulating agent, forming an emulsion or a suspension. The solvent is commonly a hydrocolloid such as gelatine, vegetable gum, modified starch, dextrin, or non-gelling protein. The solution that is obtained is dried, providing a barrier to oxygen and aggressive agents [54].

In the spray-drying process a liquid mixture is atomized in a vessel with a single-fluid nozzle, a two-fluid nozzle or spinning wheel (depending of the type of spray dryer in use) and the solvent is then evaporated by contacting with hot air or other gas. Most of spray dryers used in food industry are concurrent in design, i.e. product enters the dryer flowing in the same direction as the drying air. The objective is to obtain a very rapid drying and to avoid that the temperature of the material dried exceeds the exit air temperature of the dryer (Figure 1).
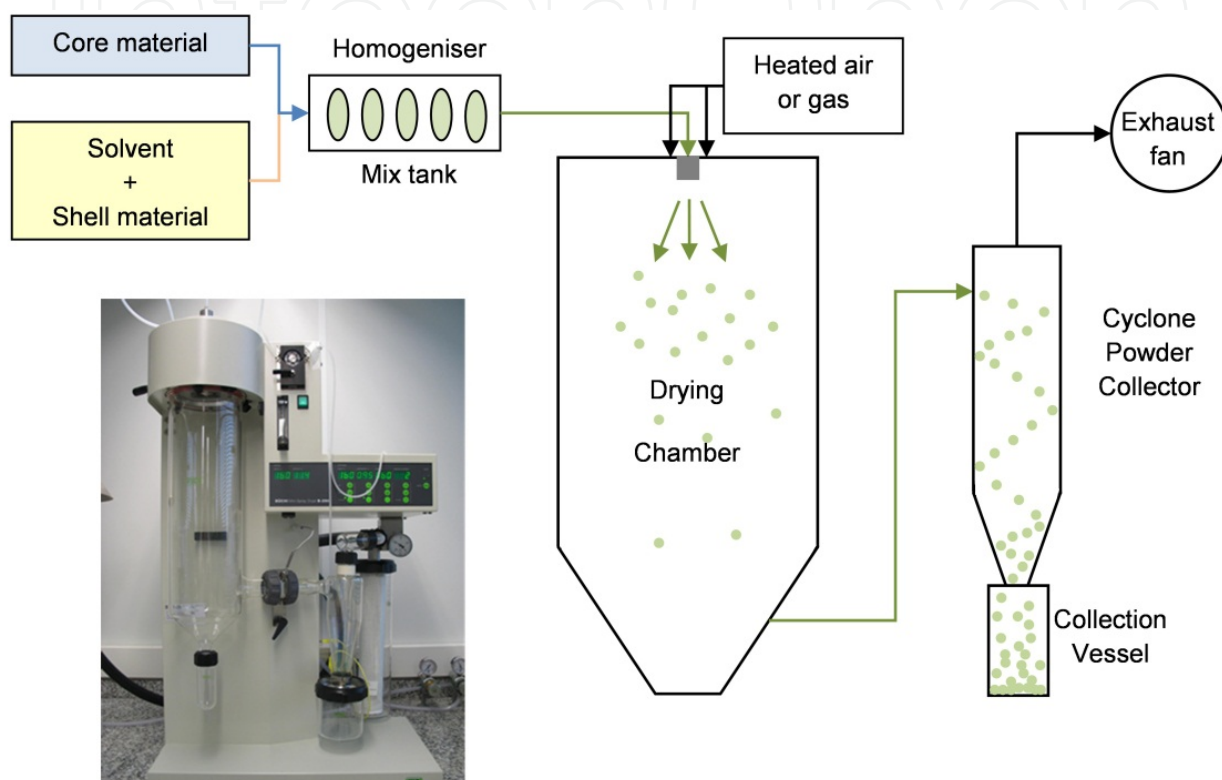
Figure 1. Schematic diagram of a spray-dry encapsulation process and image of a Mini Spray Dryer B-290 (BÜCHI), available at TECNALIA.

But also in a concurrent design, the conventional procedure requires to expose cells to high temperature and osmotic stresses due to dehydration witch results in relatively high viability and activity losses immediately after spraying and most likely also affects storage stability. However, some strains survive better than others. And parameters as drying temperature and time and shell material have also an important effect.

Using gelatinised modified starch as a carrier material, O'Riordan obtained good results in *Bifidobacterium* cells encapsulation with an inlet temperature of 100 ºC and oulet temperature of 45 ºC. Inlet temperatures of above 60 °C resulted in poor drying and the sticky product often accumulated in the cyclone. Higher inlet temperatures (>120 °C) resulted in higher outlet temperatures (>60 °C) and significantly reduced the viability of encapsulated [55]. The logarithmic number of probiotics decreases linearly with outlet air temperature of the spray-drier (in the range of 50 ºC - 80 ºC) [56]. So, the optimal outlet air temperature might be as low as possible, enough to assure the drying of the product and to

avoid the sticky effect. Alternatively, a second draying step might be applied, using a fluid bed or a vacuum oven, for example, due to the optimal survival of probiotics is achieved with low water activity.

The successful spray drying of *Lactobacillus* and *Bifidobacterium* have previously reported for a number of different strains, including *L. paracasei* [57, 58], *Lactobacillus curvatus* [59], *L. acidophilus* [60], *L. rhamnosus* [61] and *Bifdobacterium ruminantium* [8]. Specifically, Favaro-Trindade and Grosso [6] used spray drying to encapsulate *B. lactis* and *L. acidophilus* in the enteric polymer cellulose acetate phthalate enriched with the fructooligosaccharide Raftilose1 (a prebiotic). In this work, the process was also appropriate, especially for *B. lactis* (Bb-12), since for entry temperature of 130 ºC and exit of 75 ºC, the counts in the powder and dispersion (feed) were similar; however, the *L. acidophilus* population showed a reduction of two log cycles. The atomization process and encapsulant agent cellulose acetate phthalate were effective in protecting these micro-organisms in acidic medium (hydrochloric acid solutions pH 1 and 2) during incubation for up to 2 h. In another study, *B. longum* B6 and *B. infantis* were encapsulated by spray drying, with gelatin, soluble starch, milk and gum arabic as encapsulating agents. Bifidobacteria in the encapsulated form showed a small reduction in their populations when exposed to acidic media and bile solutions when compared with those exposed in the free form. Among the encapsulants tested, gelatin and soluble starch were the most effective in providing protection to the micro-organisms in acidic medium and milk was the least effective [9]. Desmond and collaborators [57] encapsulated *L. acidophilus* in β-cyclodextrin and gum arabic. They used the spray drying process, in which entry and exit temperatures of 170 ºC and 90–85 ºC respectively, and observed a reduction of 2 log cycles in the microbial population. However, the microencapsulation process extended the shelf-life of the culture.

On the other hand, the most typical materials used as carrier in probiotic bacteria encapsulation are proteins and/or carbohidrates, which may be in the glassy state at storage temperatures to minimize molecular mobility and thus degradation. The presence of some prebiotics in the encapsulating material show higher count after spray drying for *Bifidobacterium*, depending of the physical properties of the prebiotic compound selected (thermoprotector effect, crystalinity, etc.) [62, 63] and a similar effect occurs for *Lactobacillus* bacteria [61, 64]. Some researchers have proposed the addition of thermo-protectants as inputs before drying with the intention of improving the resistance to the process and stability during storage [65]. In the case of Rodríguez-Huezo and collaborators [63] used a prebiotic as encapsulant ('aguamiel') and a mixture of polymers composed of concentrated whey protein, 'goma mesquista' and maltodextrin. It is important to mention that not all the compound employees were efficient protectors. In fact, Ross and collaborators [66] reported that neither inulin nor polydextrose enhanced probiotic viability of spray-dried probiotics. In another study, it was also observed that when quercetin was added together with probiotics, the microencapsulation yields and survival rates were lower than for the micro-organism without quercetin [67]. A lot of other studies have employed of spray-drying technology to encapsulate probiotic cells, as noted in the table 1.

Probiotic strain	Materials	References
L. paracasei	Skim milk	[68]
Bifidobacterium PL1	Starch	[8]
L. acidophilus B. lactis	Cellulose acetate phthalate	[4]
L. bulgaricus	Alginate	[69]
L. paracasei	Milk based medium Gum acacia	[57]
B. breve B. longum	Whey protein	[70]
B. longum B. infantis	Gelatin soluble starch gum Arabica skim milk	[71]
B. lactis B1 L. acidophilus LAC4	Pectin and casein	[11]
L. rhamnosus GG	Whey protein-maltodextrin; Whey protein-inulin	[64]
B. longum B. infantis	Gelatin Starch Skim milk Gum arabic	[9, 72]

Table 1. Examples of encapsulated probiotic bacteria by Spray-drying Technology.

In summary, spray-drying technology offers high production rates at relatively low operating costs and resulting powders are stable and easily applicable [73]. However, most probiotic strains do not survive well the high temperatures and dehydratation during the spray-drying process. Loss of viability is principally caused by cytoplasmatic membrane damage although the cell wall, ribosomes and DNA are also affected at higher temperatures [74]. It was reported that the stationary phase cultures are more resistant to heat compare to cells in exponential growth phase [61]. One approach used by a number of researchers to improve probiotic survival is the addition of protectants to the media prior to drying. For example, the incorporation of thermoprotectants, such as trehalose [75], non-fat milk solids and/ or adnitol [76], growth promoting factors including various probiotic/prebiotic combinations [77] and granular starch [78] have been shown to improve culture viability during drying and storage [79, 80].

Microencapsulation by spray-drying is a well-established process that can produce large amounts of material. Nevertheless, this economical and effective technology for protecting materials is rarely considered for cell immobilization because of the high mortality resulting from simultaneous dehydration and thermal inactivation of microorganisms.

3.1.2. Spray-cooling

This process is similar to spray-drying described before in relation with the production of small droplets. The principal difference in the spray-cooling process is the carrier material and the working conditions related with him. In the case, a molten matrix with low melting point is used to encapsulate the bacteria and the mixture is injected in a cold air current to enable the solidification of the carrier material.

It is interesting because the capsules produced in this way are generally not soluble in water. However, due the thermal conditions of the process, the spray-cooling is used rarely for probiotics encapsulation. As example of successful development, the patent US 5,292,657 [81] present the spray-cooling of probiotics in molten lipid atomized by a rotary disk in a cooling chamber. In any case, the contact time of the probiotics with the melt carrier material should remain very sort.

### 3.1.3. Fluid-bed agglomeration and coating

The fluid-bed technology evolved from a series of inventions patented by Dr. Wurster and colleagues at the University of Wisconsin Alumni Research Foundation (WARF) between 1957 and 1966 [82, 83, 84, 85]. These patents are based on the use of fluidising air to provide a uniform circulation of particles past an atomising nozzle. This nozzle is used to atomize a selected coating material (a melt product or an aqueous solution) which solidifies in a low temperature or by solvent evaporation. A proper circulation of the particles is recognised as the key to assure that all particles in the fluid-bed achieve a uniform coating. The most commonly used techniques are referred to as the bottom-spray (Wurster) fluid-bed process and the top-spray fluid-bed process (Figure 2); however, variations such as tangential-spray are also practised.
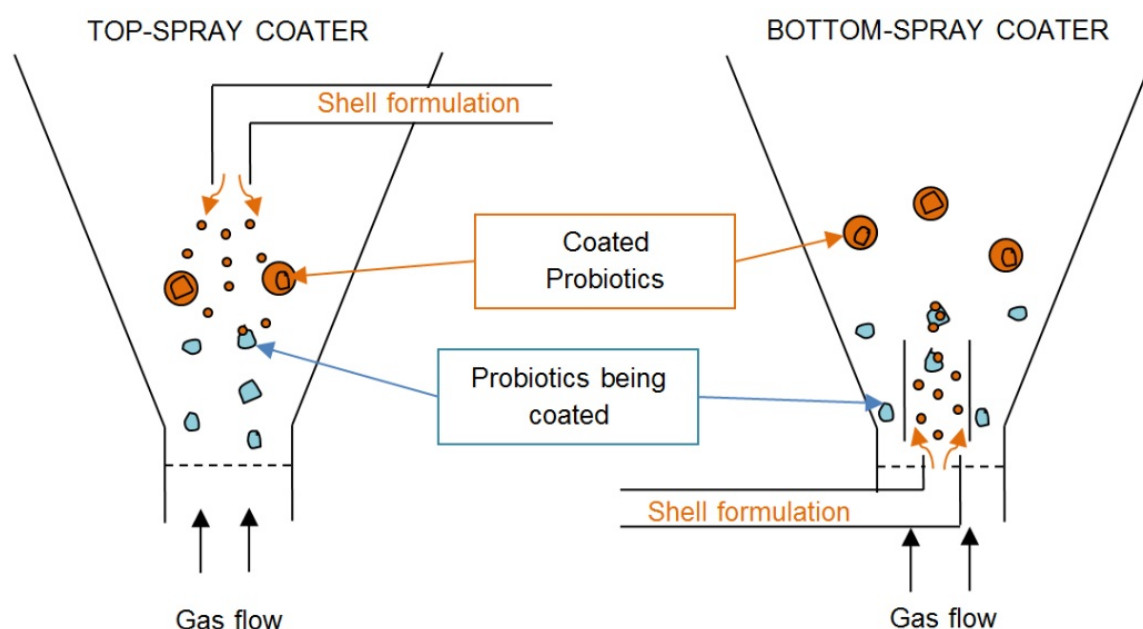
Figure 2. Schematic Diagrams of two types of the most commonly used fluid-bed coaters.

The top-spray fluid-bed coater is characterized by placement of nozzle above a fluidising bed and spraying down ware into the circulating flow of particles. This technique is useful for agglomeration or granulation. As particles flow is spray direction countercurrent, collisions involving wet particles are more probable and these collisions agglomerate particles. Bur the particles agglomerate become heavier and have less fluidization, so this phenomenon selectively agglomerates smaller particles and promotes agglomerate uniformity.

Placement of the nozzle at the bottom of a fluid bed provides the most uniform film on small particles and minimises agglomeration of such particles in the coating process compared with any other coating technique. This uniform coating is achieved because particles move further apart as they pass through the atomised spray from the nozzle and into an expansion region of the apparatus. This configuration allows the fluidising air to solidify or evaporate coating materials onto particles prior to contact between particles. A partition (centre tube) is used in Wurster fluid-bed coating to control the cyclic flow of particles in the process better than with de air distribution plate alone (Figure 3).
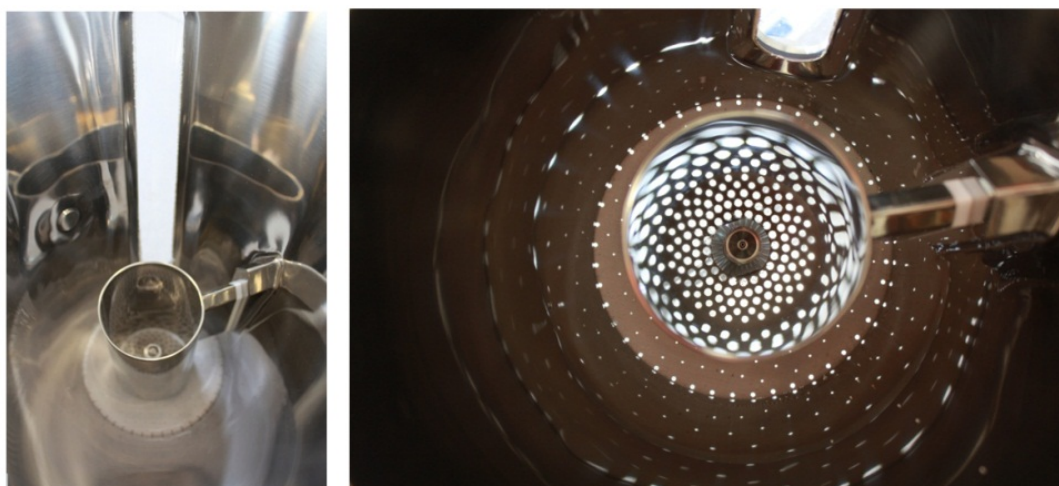
Figure 3. Expansion chamber for a bottom-spray (Wurster) fluid-bed process and detail of air distribution plate (from Glatt available at TECNALIA).

The most common coating material used for probiotics is lipid based, but proteins or carbohydrates can also be used [86]. This technique is among all, probably the most applicable technique for the coating of probiotics in industrial productions since it is possible to achieve large batch volumes and high throughputs. As example, Lallemand commercialize Probiocap™, and these particles are made in a fluid bed coating of freeze-dried probiotics with low melting lipids [87].

Specifically, Koo and collaborators [88], reported that *L. bulgaricus* loaded in chitosan-coated alginate microparticles showed higher storage stability than free cell culture. Later, Lee and researchers [69] showed that the microencapsulation in alginate microparticules coating with chitosan offers an effective way of delivering viable bacterial cells to the colon and maintaining their survival during refrigerated storage.

Fluidized-bed drying was recently investigated by Stummer and collaborators [89] as method for dehydration of *Enterococus faecium*. This study concludes to use fluidized-bed technology as a feasible alternative for the dehydration of probiotic bacteria by layering the cells on spherical pellets testing different protective agents as glucose, maltodextrin, skim milk, trehalose or sucrose, preferably skim milk or sucrose. According with the described procedure, it is possible to combine two manufacturing steps: (1) cell-dehydration preserving the optima cell properties and (2) the processing into suitable solid formulation with appropriate physical properties (the spherical pellets improve the flowability for filling capsules or dosing in different formulations.)

3.1.4. Freeze and vacuum-drying

Freeze-drying is also named lyophilisation. This drying technique is a dehydration process which works by freezing the product and then reducing the surrounding pressure to allow the frozen water to sublimate directly from the solid phase to the gas phase. The process is performed by freezing probiotics in the presence of carrier material at low temperatures, followed by sublimation of the water under vacuum. One of the most important advantages is the water phase transition and oxidation are avoided. In order to improve the probiotic activity upon freeze-drying and also stabilize them during storage, it is frequent the addition of cryoprotectans.

One of the most important aspects to decide is the choice of the optimal ending water content. This decision have to be a compromise between the highest survival rate after drying (higher survival rate with higher water content) and the lowest inactivation upon storage (better at low water activity, but not necessarily 0% of water content). According with King and collaborators [90], the loses in survival rates of freeze-dried probiotic bacteria under vacuum may be explained with a first-order kinetic and the rate constants can be described by an Arrhenius equation. But this equation might be affected by other factors as phase transition, atmosphere and water content.

In any case, the lyophilisation or freeze-drying is a very expensive technology, significantly more than spray-drying [56], even if it is probably most often used to dry probiotics. However, most of freeze-drying process only provide stability upon storage and not or limited during consumption. Because of that, this technique is used as a second step of encapsulation process. The freeze-drying is useful to dry probiotics previously encapsulated by other different techniques, as emulsion [91] or entrapment in gel microspheres [92]. In this way it is possible to improve the stability in the gastrointestinal tract and optimize the beneficial effect of probiotic consumption.

The Vacuum-drying is a similar process as freeze-drying, but it takes place at 0 - 40 ºC for 30 min to a few hours. The advantages of this process are that the product is not frozen, so the energy consumption and the related economic impact are reduced. In the product point of view, the freezing damage is avoided.

3.1.5. Emulsion-based techniques

An emulsion is the dispersion of two immiscible liquids in the presence of a stabilizing compound or emulsifier. When the core phase is aqueous this is termed a water-in-oil emulsion (w/o) while a hydrophobic core phase is termed an oil-in-water emulsion (o/w). Emulsions are simply produced by the addition of the core phase to a vigorously stirred excess of the second phase that contains, if it is necessary, the emulsifier (Figure 4). Nevertheless, even if the technique readily scalable, it produce capsules with an extremely large size distributions. Because of this limitation, there are several industrial efforts to achieve a narrow particle size distribution controlling the stirring and homogenization of the mixture.

There are also double emulsions, such water-in-oil-in-water (w/o/w). The technique is a modification of the basic technique in which an emulsion is made in of an aqueous solution in a hydrophobic wall polymer. This emulsion is the poured with vigorous agitation, into an aqueous solution containing stabilizer. The loading capacity of the hydrophobic core is limited by the solubility and diffusion to the stabilizer solution. The principal application of this technology is in pharmaceutical formulations.

Entrapment of probiotic bacteria in emulsion droplets has been suggested as a means of enhancing the viability of microorganism cells under the harsh conditions of the stomach and intestine. For example, Hou and collaborators [93] reported that entrapment of cells of lactic bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus*) in the droplets of reconstituted sesame oil body emulsions increased approximately 10^4 times their survival rate compared to free cells when subjected to simulated GI tract conditions.
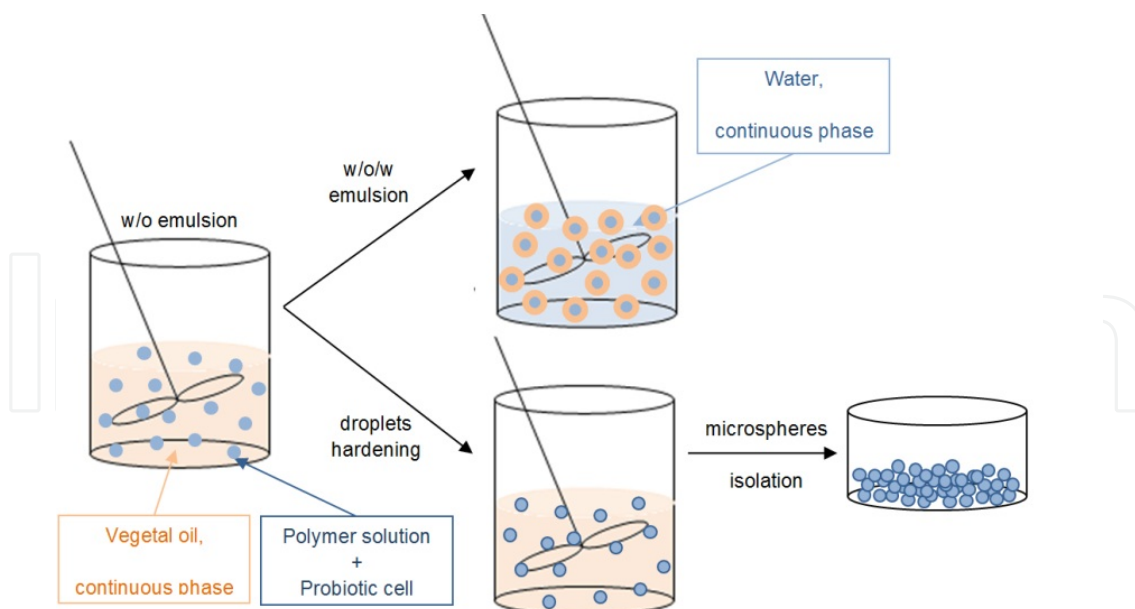
Figure 4. Probiotic cell encapsulation by water-in-oil and water-in-oil-in-water emulsions.

Nevertheless, Mantzouridou and collaborators [94] have presented an study investigating the effect of cell entrapment inside the oil droplets on viable cell count over storage and under GI simulating conditions, according to the type of emulsifier used: egg yolk, gum arabic/xanthan

mixture or whey protein isolate. The study was performed with *Lactobacillus paracasei* and their entrapment in the oil phase of protein-stabilized emulsions protected the cells when exposed to GI tract enzymes, provided that the emulsions were freshly prepared. Following, however, treatment of aged for up to 4 weeks emulsions under conditions simulating those of the human GI environment, the microorganism did not survive in satisfactory numbers. The probiotic cells survived in larger numbers in aged emulsions when the cells were initially dispersed in the aqueous phase of a yolk-stabilized dressing-type emulsion and their ability to survive enzymatic attack was further enhanced by inulin incorporation.

| Probiotic strain	Materials	References
B. bifidum	κ-Carrageenan	[96]
L. lactis spp. lactis	κ-Carrageenan	[97]
L. acidophilus B. infantis	Alginate/starch	[98]
L. acidophilus B. bifidum	Alginate	[99, 100]
B. longum	κ-Carrageenan	[101]
L. acidophilus	Alginate-starch	[102]
B. adolescentis	Alginate	[103
B. longum	κ-Carrageenan	[104
L. acidophilus B. infantis	Alginate/starch	[105]
B. breve	Milk fat and whey protein	[70]
L. acidophilus L. casei L. rhamnosus B. infantis	Alginate	[106]
L. acidophilus B. bifidum	Alginate/starch	[52]
L. bulgaricus	Alginate	[107]
L. casei B. lactis	Alginate	[108]
L. acidophilus B. infantis	Alginate/starch	[109]
L. acidophilus B. lactis		[110]
L. casei B. lactis	Ca-alginate	[111]
B. bifidum B. infantis	Alginate	[112]
L. rhamnosus L. salivarius B. longum L. plantarum L. acidophilus L. paracasei B. lactis		[113]
L. acidophilus B. infantis	Alginate/starch	[109]
L. acidophilus B. lactis		[110]
L. delbrueckii ssp. bulgaris		[93]
B. pseudolongum	Cellulose acetate phthalate	[114]
L. casei ssp. casei	κ-Carrageenan/locust bean gum	[115]
B. longum Lactococcus lactis	κ-Carrageenan/locust bean gum	[116]
B. lactis	Gelatin	[117]

Table 2. Examples of encapsulated probiotic bacteria by Emulsification Technology.

Lactobacillus rhamnosus has been encapsulated in a w/o/w emulsion. According to Pimentel-González and collaborators [95] the survival of the entrapped *L.rhamnosus* in the inner water phase of the double emulsion increased significantly under low pH and bile salt conditions in an *in vitro* trial, meanwhile the viability and survival of control cells decrease significantly under the same conditions.

In the table 2 details probiotic strains and carrier materials that have employed some researchers in the emulsification technology.

The emulsion methods produce capsules sized from a few micrometres to 1mm, approximately, but with a high dispersion compared to other techniques, as extrusion ones. Moreover, even if the emulsion techniques described before are easily scalable, these techniques have an important disadvantage to be applied in an industrial process because are batch processes. Nevertheless, it exist another promising technique different to the turbine used. The static mixers are small devices placed in a tube consisting in static obstacles or diversions where the two immiscible fluids are pumped [118, 119]. This system improves the size distribution, reduce shear and allows keeping the aseptic conditions because it might be a closed system (Figure 5). For example, nowadays this technology is used in dairy industry for viscous products, as admixing fruit pieces or cultures to yoghurt or to process ice cream or curds.
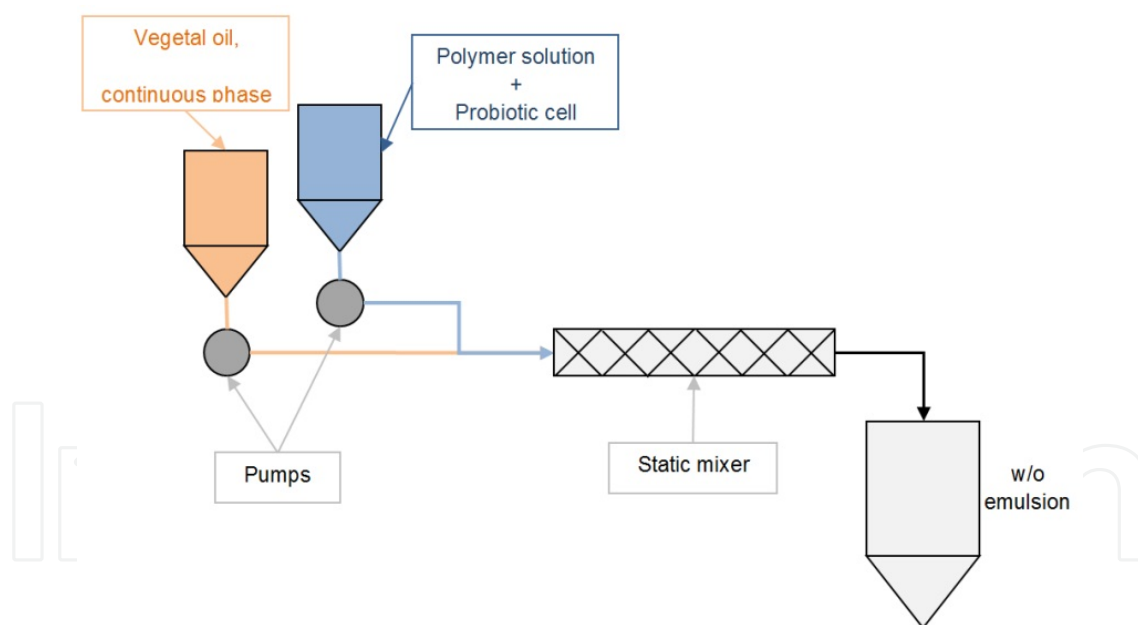
Figure 5. Schematic diagram of a static mixer system to make emulsions.

3.1.6. Coacervation

This process involves la precipitation of a polymer or several polymers by phase separation: simple or complex coacervation, respectively. Simple coacervation is based on "salting out" of one polymer by addition of agents as salts, that have higher affinity to water than the polymer. It is essentially a dehydration process whereby separation of the liquid phase

results in the solid particles or oil droplets (starting in an emulsion process) becoming coated and eventually hardened into microcapsules. With regard to complex coacervation, it is a process whereby a polyelectrolyte complex is formed. This process requires the mixing of two colloids at a pH at which both polymers are oppositely charged (i.e. gelatine (+) and arabic gum (-)), leading to phase separation and formation of enclosed solid particles or liquid droplets.

The complex coacervation is one of the most important techniques used for flavour microencapsulation. But it is not the only use of this technique and the complex coacervation is also suitable for probiotic bacteria microencapsulation. And the most frequent medium used might be a water-in-oil emulsion [120].

Oliveira and collaborators [121] encapsulated *B. lactis* (BI 01) and *L. acidophilus* (LAC 4) through complex coacervation using a casein/pectin complex as the wall material. To ensure higher stability, the coacervated material was atomized. The process used and the wall material were efficient in protecting the microorganisms under study against the spray drying process and simulated gastric juice; however, microencapsulated *B. lactis* lost its viability before the end of the storage time. Specifically, microencapsulated *L. acidophilus* maintained its viability for a longer storage (120 days) at 7 and 37 ºC, *B. lactis* lost viability quickly.

Advantages of coacervation, compared with other methods for the encapsulation of probiotics, are a relatively simple low-cost process (which does not necessarily use high temperatures or organic solvents) and allow the incorporation of a large amount of micro-organisms in relation to the encapsulant. However, the scale-up of coacervation is difficult, since it is a batch process that yields coacervate in an aqueous solution. Therefore, to extend its shelf-life, an additional drying process should be applied, which can be harmful to cells.

### 3.1.7. Extrusion techniques to encapsulate in microspheres

The methods of bioencapsulation in microspheres include two principal steps: (1) the internal phase containing the probiotic bacteria is dispersed in small drops a then (2) these drops will solidify by gelation or formation of a membrane in their surface. Before this section, there are described emulsion systems and coacervation as different methods to obtain these drops and even the membrane formation, but also extrusion technology is useful in order to produce probiotic encapsulation in microspheres. There are different technologies available for this purpose and the selection of the best one is related with different aspects as desired size, acceptable dispersion size, production scale and the maximum shear that the probiotic cells can support.

When a liquid is pumped to go through a nozzle, first this is extruded as individual drops. Increasing enough the flow rate, the drop is transformed in a continuous jet and this continuous jet has to be broken in small droplets. So, the extrusion methods could be divided in two groups, dropwise and jet breakage (Figure 6), and the limit between them is established according to the minimum jet speed according to this equation (eq. 1):

$$v_{j,min} = \text{minimum jet speed } (m/s)$$

$$v_{j,min} = 2\left(\sigma / d_j\right)^{0,5} \qquad \sigma = \text{surface tension } (N/m) \qquad (1)$$

$$\rho = \text{liquid flowing density } (kg/m^3)$$

$$d_j = \text{jet diameter } (m)$$

Regardless of the selected technique, the liquid obtained drops have to be solidifying by gelation or external membrane formation (Figure 6). The resulting hydrogel beads are very porous and a polymeric coating is usually applied in order to assure a better retention of the encapsulated probiotic bacteria.
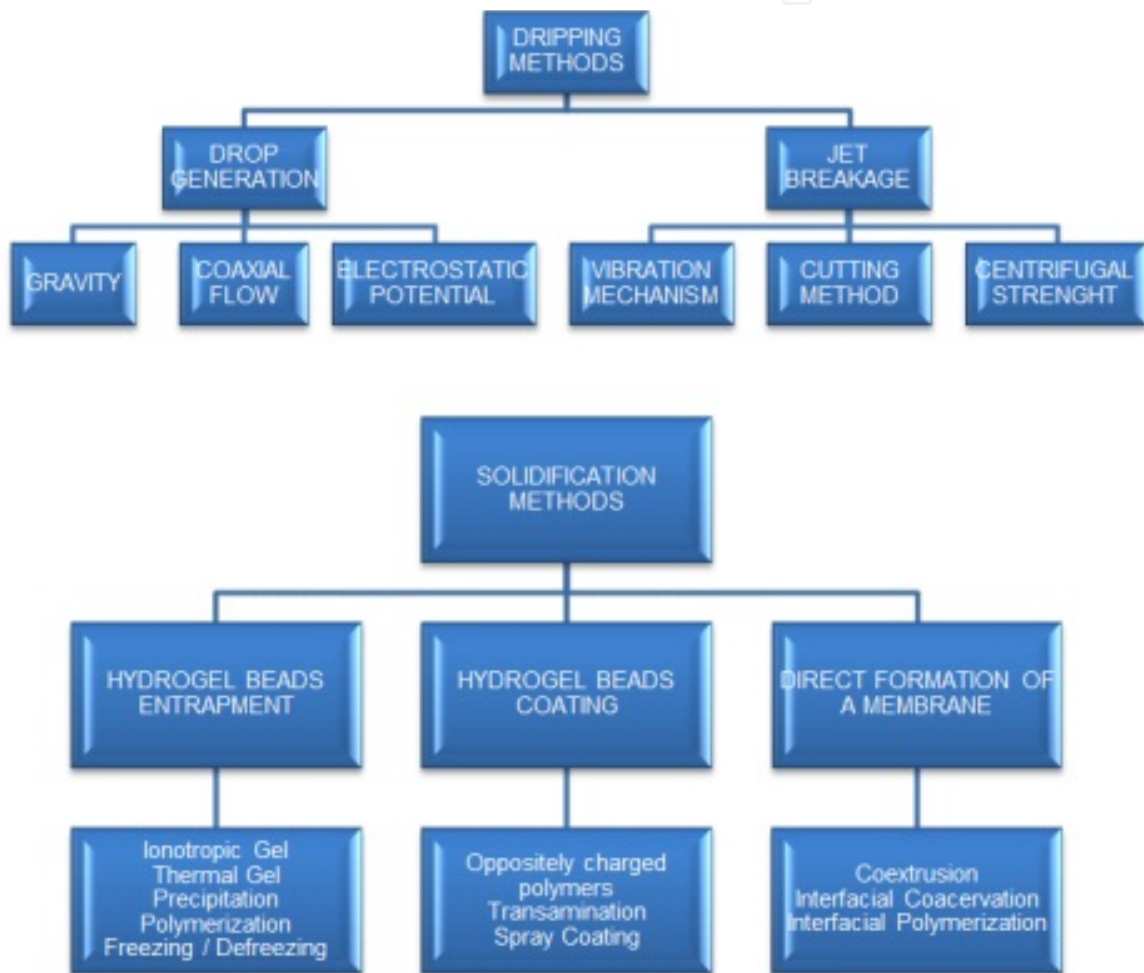
Figure 6. Classification of methods to make and solidify drops

- **Dripping by gravity**

This method is the simplest dripping method to make individual drops, but the size of the droplet will be determined by his weight and surface tension, as well as the nozzle perimeter. The typical diameter of a drop made by this technique is higher than 2 mm. Moreover, the flow is around several millilitres by hour and the method is not interesting for an industrial application. For example, in the Figure 7 is showed a cell immobilization

process carried out at TECNALIA using the method of dripping by gravity. The nozzle diameter is 160 µm and the final size of hydrogel bead (after solidification in a Calcium Chloride solution) is 2,4±0,15 mm.
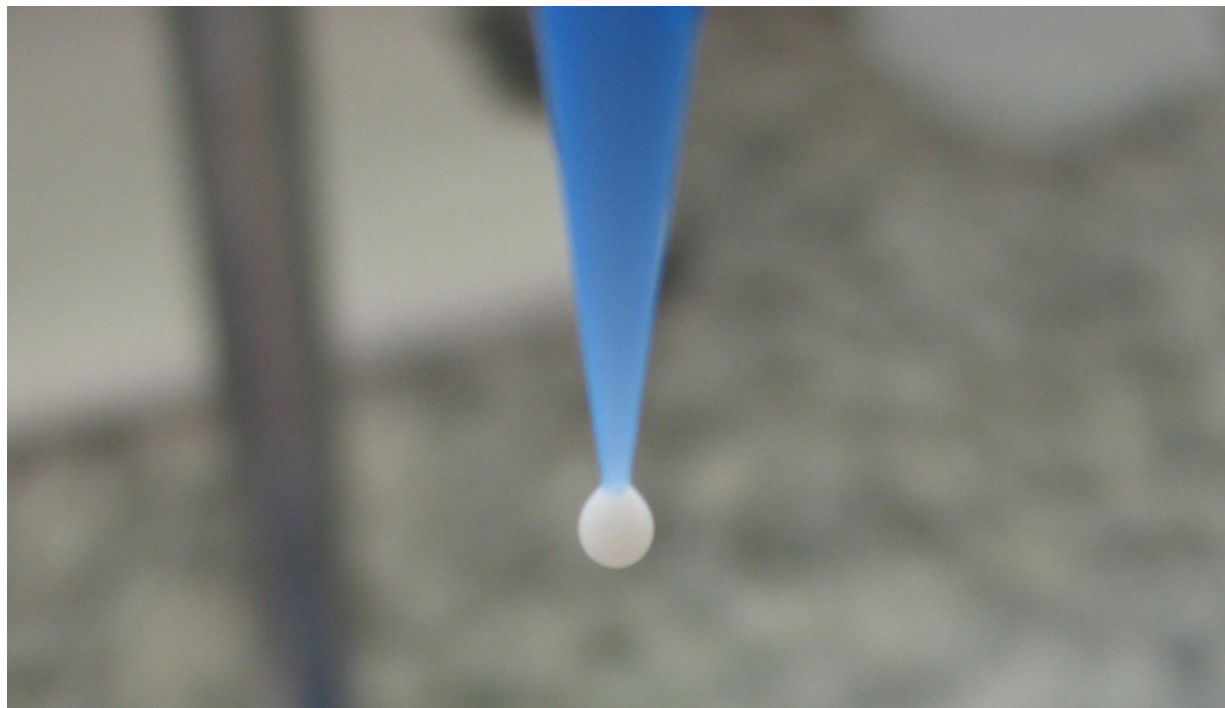

Figure 7. Cell encapsulation in an alginate matrix. Drop generation by gravity using a 160 µm nozzle.

- **Air o liquid coaxial flow and submerged nozzles**

Applying a coaxial air flow around the extrusion nozzle it is possible to reduce the microsphere diameter between a few micrometres and 1 mm. However, the flow rate is limited, less than 30 mL/h to avoid a continuous jet formation. The air flow might be replaced for a liquid one: with a suitable selection of the liquid flow the control of the surface tension is improved. Drops produced in air are generated as aerosols, while the drops produced, for example, in water are made as emulsions. The aerosol beads could be solidified using ionic gelation or hot air. The beads recovered as emulsion are usually extracted or the water is evaporated.

The Spanish enterprise Ingeniatrics Tecnologías has patent an owner Flow Focusing® technology, valid to work with air and liquid flow, and also an user-friendly bioencapsulation device for biotechnological research and clinical microbiology able to encapsulate high molecular weight compounds, microorganisms and cells in homogeneous particles of predictable and controllable size based on Flow Focusing® technology named Cellena® distributed by Biomedal (Figure 8).

Nevertheless, despite all the advantages, due to the mentioned low flow rate, this technique is not used in an industrial scale and also in a laboratory scale it is being replaced for the jet breakage techniques stated below.
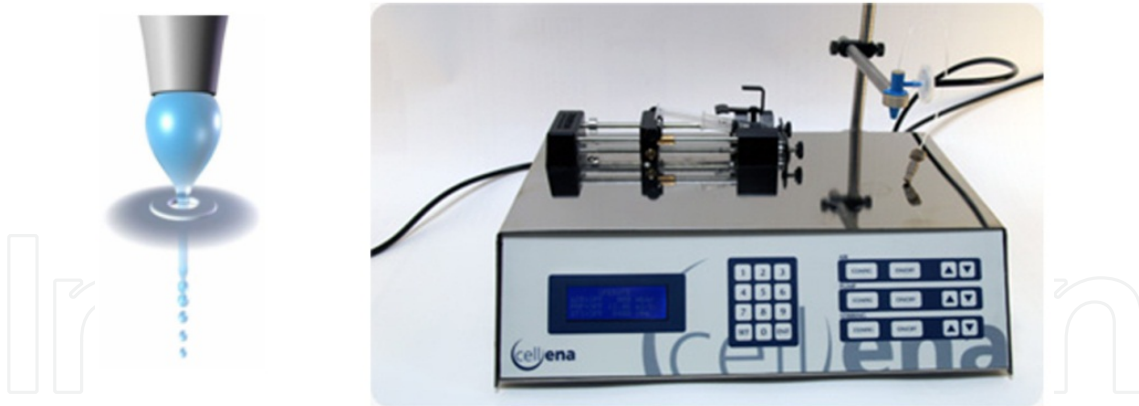
Figure 8. Flow-Focusing technology to make droplets and Cellena® equipment from Ingeniatrics Tecnologías.

The submerged nozzles usually are static, but they can be also rotating or vibrating to improve the droplet generation, but are always immersed in a carrier fluid. An example of the former consist of a static cup immersed in a water-immiscible oil such as mineral oil or vegetal oil and a concentric nozzle as is schematically showed in the Figure 9. Each droplet consist of core material being encapsulatd totally surrounded by a finite film of aqueous polymer solution, as gelatine, for example. The carrier fluid, a warm oil phase that cools after droplet formation, gels this polymer solution thereby forming gel beads with a continuous core/shell structure. The smaller diameter using this technique is typically around 1 mm.
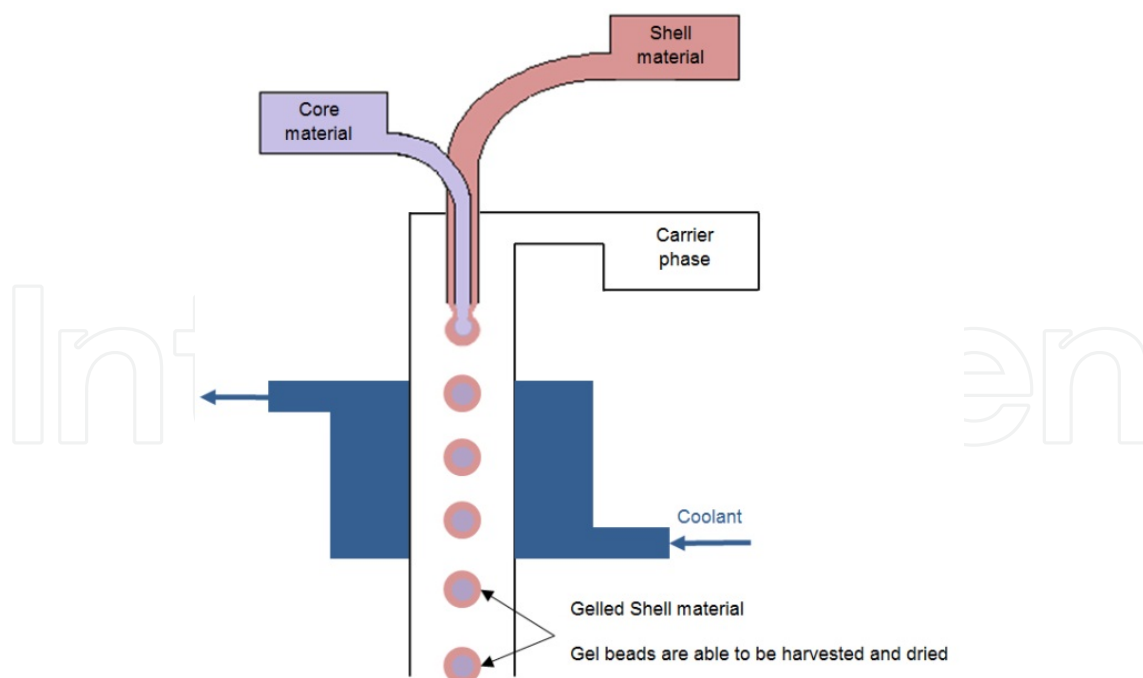
Figure 9. Schematic diagram of a submerged two-fluid static nozzle.

An example of this technology is provided by Morishita Jintan Co. Ltd in Japan These capsules are composed of three layers: a core freeze-dried probiotic bacteria in solid fat, with

an intermediate hard fat layer and a gelatin-pectin outer layer [122]. However, the size of the capsules produced is quite large to be applied in food products (1.8-6.5 mm) and the technique is quite expensive for use in many food applications.

- **Electrostatic potential**

This technique is the last one of drop generation techniques. The droplet generation improves replacing the dragging forces by a high electrostatic potential between the capillary nozzle and the harvester solution. The electric forces help the gravity force in front of the surface tension.

Even if the capsules size is appropriated and the size distribution is narrow enough, this technique is more expensive than other extrusion ones and it is not fast enough to be scaled.

- **Vibration technology for jet break-up**

Applying a vibration on a laminar jet for controlled break-up into monodisperse microcapsules is one among different extrusion technologies for encapsulation of probiotic bacteria. The vibration technology is based on the principle that a laminar liquid jet breaks up into equally sized droplets by a superimposed vibration (Figure 10). The instability of liquid jets was theoretically analysed for Lord Rayleigh [123]. He showed that the frequency for maximum instability is related to the velocity of the jet and the nozzle diameter (eq. 2 and eq. 3).

$$f_{opt} = {v_J}/{\lambda_{opt}} \qquad \begin{array}{l} f_{opt} = \text{optimal frequency } (\text{Hz}) \\ v_J = \text{jet velocity } (\text{m}/\text{s}) \\ \lambda_{opt} = \text{optimal wavelength } (\text{m}) \end{array} \qquad (2)$$

$$\lambda_{opt} = \pi\sqrt{2}d_N\sqrt{1 + \frac{3}{\sqrt{\rho\sigma d_N}}} \qquad \begin{array}{l} d_N = \text{nozzle diameter } (\text{m}) \\ \eta = \text{dynamic viscosity } (\text{kg}/\text{m s}) \\ \rho = \text{density } (\text{kg}/\text{m}^3) \\ \sigma = \text{Surface tension } (\text{N}/\text{m}) \end{array} \qquad (3)$$

Using this technology, it is possible to obtain monodisperse droplets which size can be freely chosen in a certain range depending on the nozzle diameter and the frequency of the sinusoidal force applied (eq. 4). The droplets made are harvested in an accurate hardening bath. To avoid large size distributions due to coalescence effects during the flight and the hitting phase at the surface of hardening solution the use of a dispersion unit with an electrostatic dispersion unit is essential (Figure 10).

$$d_D = \sqrt[3]{1.5 d_N^2 \lambda_{opt}} \qquad \begin{array}{l} d_D = \text{droplet diameter } (\text{m}) \\ d_N = \text{nozzle diameter } (\text{m}) \\ \lambda_{opt} = \text{optimal wavelength } (\text{m}) \end{array} \qquad (4)$$
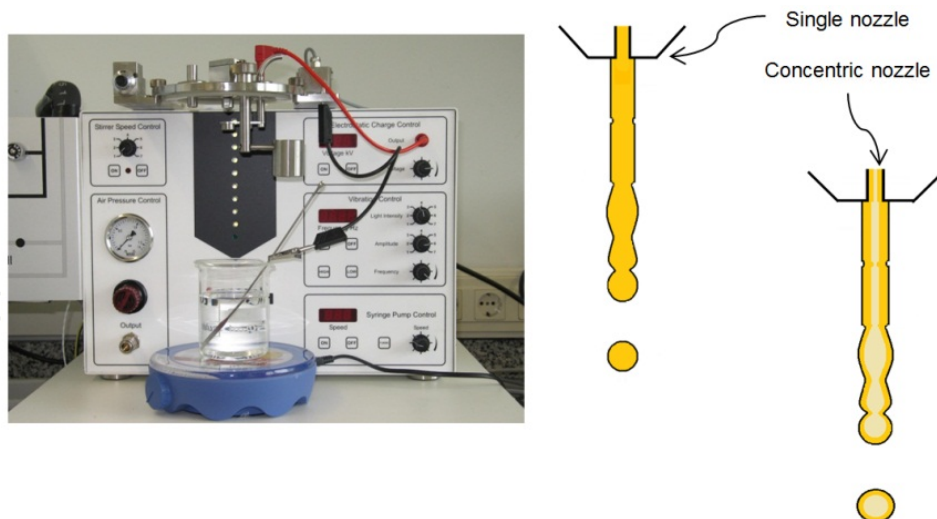
Figure 10. Image of Inotech Encapsulator IE-50R and schematic diagram of jet destabilization and breakage for single and concentric nozzles.

The Encapsulator BIOTECH (the updated version of IE-50R) from EncapBioSystems and Spherisator form BRACE GmbH are two different devices labels to produce microencapsuled probiotic bacteria using the vibration technology for jet breakage. The principal advantages of this technology are the low size dispersion (5-10%), a high flow rate (0.1-2 L/h) and is able to work in sterile conditions. The possibility of working with a wide range of materials (hot melt products, hydrogels, etc.) is also an important aspect to be considered, as well as the design with also concentric nozzles in the lab scale devices and with this kind of nozzles it is possible to produce capsules with a defined core region (solid or liquid) surrounded by a continuous shell layer. On the other side, the principal disadvantage of this technology is the limit in the viscosity for the liquid to be extruded.

But may be one of the most important advantage of the vibration devices commercialized is that the scale up of this technology is relatively "simple" and it consist in the multiplication of the number of nozzles, developing multinozzle devices. The only challenge is that each nozzle of a multinozzle plant must operate in similar production conditions: equal frequency and amplitude, and equal flow rate. In this way, the scale up is direct from the lab to a pilot or industrial scale.

- **JetCutter technology**

The bead production by JetCutter (from geniaLab) is achieved cutting a jet into cylindrical segments by a rotating micrometric cutting tool. The droplet generation is based on a mechanical impact of the cutting wire on the liquid jet. Some techniques as emulsion, simple dropping, electrostatic-enhanced dropping, vibration technique or rotating disc and nozzle techniques have in common that the fluids have to be low in viscosity, and not all of them may be used for large-scale applications. On the contrary, the JetCutter technique is especially capable of processing medium and highly viscous fluids up to viscosities of several thousand mPas.

For bead production by the JetCutter the fluid is pressed with a high velocity out of a nozzle as a solid jet. Directly underneath the nozzle the jet is cut into cylindrical segments by a rotating cutting tool made of small wires fixed in a holder (Figure 11). Driven by the surface tension the cylindrical segments form spherical beads while falling further down, where they finally can be harvested. The size of beads can be adjusted within a range between approximately 200 μm up to several millimetres, adjusting parameters as nozzle diameter, flow rate, number of cutting wires and the rotating speed of cutting tool.

Bead generation by a JetCutter device is achieved by the cutting wires, which cut the liquid jet coming out of the nozzle. But in each cut the wire produce a cutting loss. The device is designed to recover these losses, but it is important to minimize de lost volume selecting a smaller diameter of the cutting wire and angle of inclination of the cutting tool with regard to the jet (Figure 11). According with Pruesse and Vorlop [124], a suitable model of the cutting process might help to operator in the parameters selection. One of the most important parameters is the ratio of the velocities of the fluid and cutting wire, necessary to determinate the proper inclination angle (eq. 5), but the fluid velocity is also related with the bead size (eq. 6), while the diameter of the nozzle and wire determine the volume of cutting loses (eq. 7).

$$\alpha = arcsin\left(u_{fluid} \middle/ u_{wire} \right)$$

α = inclination angle
u_{fluid} = velocity of the fluid
u_{wire} = velocity of the cutting wire

(5)

$$d_{bead} = \sqrt[3]{\frac{3}{2} \cdot D^2 \cdot \left(\frac{u_{fluid}}{n \cdot z} - d_{wire} \right)}$$

d_{bead} = bead diameter
D = nozzle diameter
d_{wire} = cutting wire diameter

(6)

$$V_{loss} = \frac{\pi \cdot D^2}{4} \cdot d_{wire}$$

n = number of rotations
z = number of cutting wires
V_{loss} = Volume of the overall loss

(7)

Regarding the advantages of the JetCutter technology, besides the capacity for work with medium and highly viscous fluids, there are the narrow bead size dispersion and the wide range of possible sizes, as well as the high flow rate (approx. 0.1-5 L/h).

To scale up the JetCutter technology there are two ways. First, a multi-nozzle device can be used, in which nozzles are strategically distributed in the perimeter of the cutting tool. The second way is the increase of the cutting frequency, but this approach needs also a higher velocity of the jet and a too high speed of the beads might cause problems, as coalescence or deformation in the collection bath entrance. In order to overcome this problem, the droplets can be pre-gelled prior entering the collection bath using, for example, a tunnel equipped with nozzles spraying the hardening solution or refrigerating the falling beads.

The extrusion technique is the most popular microencapsulation or immobilization technique for micro-organisms that uses a gentle operation which causes no damage to probiotic cells

and gives a high probiotic viability [21]. This technology does not involve deleterious solvents and can be done under aerobic and anaerobic conditions. The most important disadvantage of this method is that it is difficult to use in large scale productions due to the slow formation of the microbeads [15]. Various polymers can be used to obtain capsules by this method, but the most used agents are alginate, κ-carrageenan and whey proteins [125].
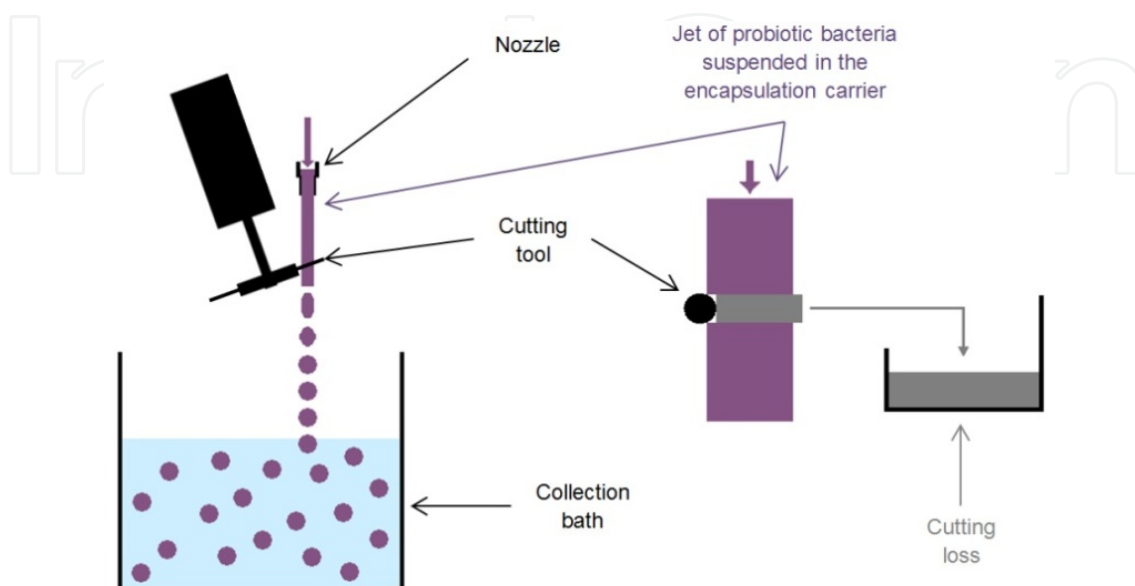
Figure 11. Schematic diagram of the JetCutter technology and representation of fluid losses due to the cutting wire impact.
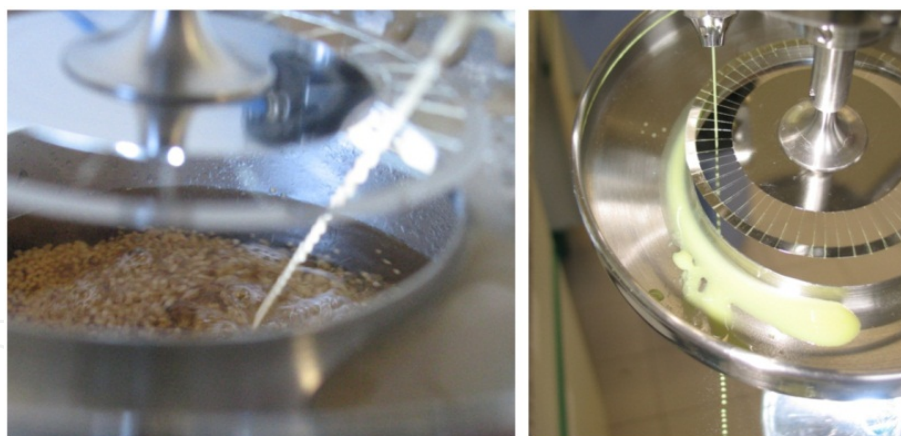
Figure 12. Examples of two bioencapsulation process carried out at TECNALIA changing the nozzle diameter, cutting tool and inclination angle to obtain different bead size necessaries for several applications.

There are many studies with the extrusion techniques for probiotic protection and stabilization. In 2002, Shah and Ravula [126] encapsulated *Bifidobacterium* and *Lactobacillus acidophilus* in calcium alginate in frozen fermented milk-based dessert, and, in general, the survival of bacteria cells was improved by encapsulation. Some studies employed to encapsulate Bifidobacteria alginate alone and a mixture with other compounds and observed more resistant to the acidic medium than the free cells [5, 112, 127]. A similar

result was observed by Chávarri and collaborators [67], where chitosan was used as coating material to improve the stability of alginate beads with probiotics. In this study, with extrusion technique, they showed an effective means of maintaining survival under simulated human gastrointestinal conditions. In the table 3 details probiotic strains and carrier materials that have employed some researchers in the extrusion technology.

Probiotic strain	Materials	References
L. casei		[128]
B. infantis	Gellan/xantan gum	[129]
L. acidophilus	Raftilose, raftiline and Storch	[130]
L. acidophilus B. infantis	Ca-alginate	[131]
L. acidophilus	Ca-alginate Chitosan	[132]
L. acidophilus B. bifidum L. casei	Alginate-chitosan	[133]
L. acidophilus L. acidophilus	Alginate-chitosan	[134]
B. lactis		[135]
L. acidophilus B. lactis		[136]
L. casei	Alginate/pectin	[137]
L. bulgaricus S. thermophilus	Ca-alginate	[138]
L. acidophilus B. bifidum	Alginate	[99, 100]
L. lactis	Ca-alginate	[139]
L. acidophilus	κ-Carrageenan	[140]
L. gasseri B. bifidum	Alginate-chitosan	[67]
B. lactis	Gellan/Xanthan gum	[141]
L. reuteri	Alginate	[142, 143]
L. rhamnosus	Whey protein	[144]
L. acidophilus	Ca-alginate	[145]
L. acidophilus	Ca-alginate	[140]
L. acidophilus B. bifidum	Alginate	[99, 100]
L. lactis		[139]
B. longum	Alginate	[146]
L. acidophilus B. longum	Alginate	[126]
B. bifidum B. infantis	Alginate	[147]
Lactococcus lactis ssp. cremoris	Alginate	[148]
L. rhamnosus GG	Whey protein Gellan gum	[150]
L. plantarum L. rhamnosus	Xanthan gum Pullulan gum Jamilan	[150]
L. acidophilus B. lactis	Alginate	[151]

Table 3. Examples of encapsulated probiotic bacteria by Extrusion Technology.

3.1.8. Adhesion to starch granules

Starch is unique among carbohydrates because it occurs naturally as discrete particles called granules. Their size depends on the starch origin ranging from 1 to 100 μm. They are rather dense and insoluble, and hydrate only slightly in water at room temperature. The granular structure is irreversibly lost when the granules are heated in water about 80 ºC, and heat and mechanical energy are necessaries to totally dissolve the granules.

Usually starches are partially or totally dissolved before they are used in food application, for example to be used as texturizing. Starch hydrolysates or chemically modified starches are used as microencapsulation matrices for lipophilic flavours [152, 153]. Partially hydrolysed and crosslinked starch granules were suggested to be suitable carriers for various functional food components [154]. To hydrolyse the starch granules, the use of amylases is the preferred way and corn starch seems to be the most suitable starch for his purpose.

Some probiotic bacteria were shown to be able to adhere to starch and a few investigations about the utilisation of starch granules to protect these bacteria were reported.

3.1.9. Compression coating

This technique involves compressing dried bacteria powder into a core tablet or pellet and the compressing coating material around the core to form the final compact (Figure 13). The compression coating has received a renewed interest for probiotic bacteria encapsulation used with gel-forming polymers in order to improve the stabilization of lyophilized bacteria during storage [155]. The viability in process of the bacteria is affected by the compression pressure and to improve the storage survival the coating material has a significant effect.

Due of the size of the final product obtained by compression coating, this technique is used for pharmaceutical and nutraceutical compounds development, but not for food ingredients obtention.
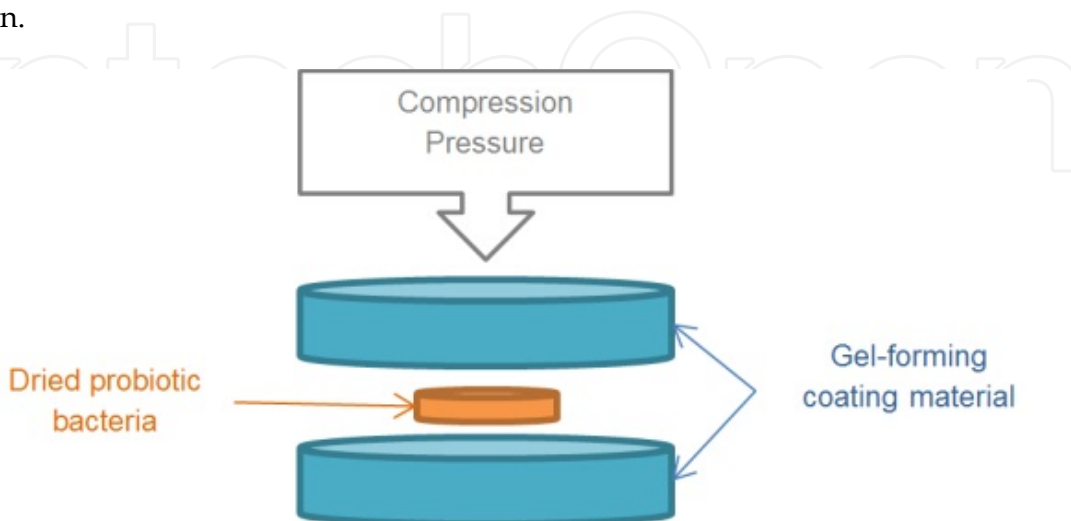
Figure 13. Schematic diagram of compression coating of probiotics.

3.2. Shell or carrier encapsulation materials

Microcapsules should be water-insoluble to maintain their structural integrity in the food matrix and in the gastrointestinal tract. The materials are used alone or in combination to form a monolayer. In this last case, coating the microcapsule with the double membrane can avoid their exposure to oxygen during storage and can enhance the resistance of the cells to acidic conditions and higher bile salt concentrations.

3.2.1. Ionic hydrogels

- **Alginate**

Alginate is surely the biopolymer most used and investigated for encapsulation. Alginates are natural occurring marine polysaccharides extracted from seaweed, but also they occur as capsular polysaccharides in some bacteria [156]. Being a natural polymer, alginic acids constitute a family of linear binary copolymers of 1-4 glycosidically linked α-L-guluronic acid (G) and its C-5 epimer β-D-mannuronic acid. (M). Alginates are the salts (or esters) of these polysaccharides. They are composed of several building blocks (100-3,000 units) liked together in a stiff and partly flexible chain. The relative amounts of the two uronic units and the sequential arrangements of them along the polymer chain vary widely, depending of the origin of the alginate: three types of blocks may be found: homopolymeric M-blocks (M-M), homopolymeric G-blocks (G-G) and heteropolymeric sequentially alternating MG-blocks (M-G). This composition and block structure are strongly related to the functional properties of alginate molecules within an encapsulation matrix.

Immobilisation or entrapment of probiotic bacteria in alginate it is possible due to it is a rapid, non-toxic and versatile method for cells. Dissolving alginate in water gives a viscous solution of which the viscosity will increase with the length of the macromolecule (number of monomeric units), and its solubility is also affected by the pH (at pH < 3 precipitate as alginic acid), the presence of counterions in water (alginate precipitates by crosslinking, gelling, with divalent ions such as Ca^{2+}, Ba^{2+}, Sr^{2+}…) and the sequential arrangements of the monomers (the flexibility of the alginate chains in solution increases in the order MG<MM<GG). The gelling occurs when a cation as Ca^{2+} take part in the interchain binding between G-bloks giving rise to a three-dimensional network (Figure 14).

The advantage of alginate is that easily form gel matrices around bacterial cells, it is safe to the body, they are cheap, mild process conditions (such as temperature) are needed for their performance, can be easily prepared and properly dissolve in the intestine and release entrapped cells. However, some disadvantages are attributed to alginate beads. For example, alginate microcapsules are susceptible to the acidic environment [136] which is not compatible for the resistance of the beads in the stomach conditions. Other disadvantage of alginate microparticle is that the microbeads obtained are very porous to protect the cells from its environment [157]. Nevertheless, the defects can be compensated by blending of alginate with other polymer compounds, coating the capsules by another compound or structural modification of the alginate by using different additives [21].
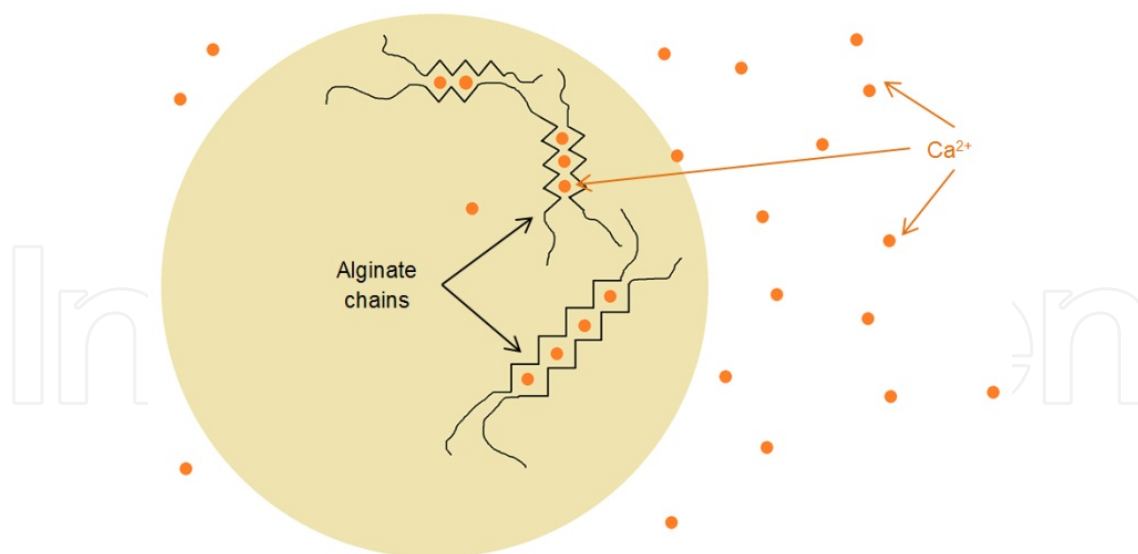
Figure 14. Gelation of an alginate bead when the Ca^{2+} gelling ions diffuse into the alginate-containing system.

- **Chitosan**

Chitosan is a deacetylated derivative of chitin, which is widely found in crustacean shells, fungi, insects and molluscs. This polymer is a linear polysaccharide, which can be considered as a copolymer consisting of randomly distributed β-(1,4) linked D-glucosamine and N-acetyl-D-glucosamine. The functional properties of chitosan are determined by the molecular weight, but also by the degree of acetylation (DA), which represents the proportion of N-acetyl-D-glucosamine units with respect to the total number of units [158]. Chitosan is soluble in acidic to neutral media, but solubility and viscosity of the solution is dependent on the length of chains and the DA.

As chitosan is a positively charged polymer, it forms ionic hydrogels by addition of anions such as pentasodium tripolyphosphate (TPP) and also by interaction with negatively charged polymers as alginate [67] or xanthan [159]. It is possible to obtain an hydrogel by precipitation in a basic medium or by chemical crosslinking with glutaraldehyde [160].
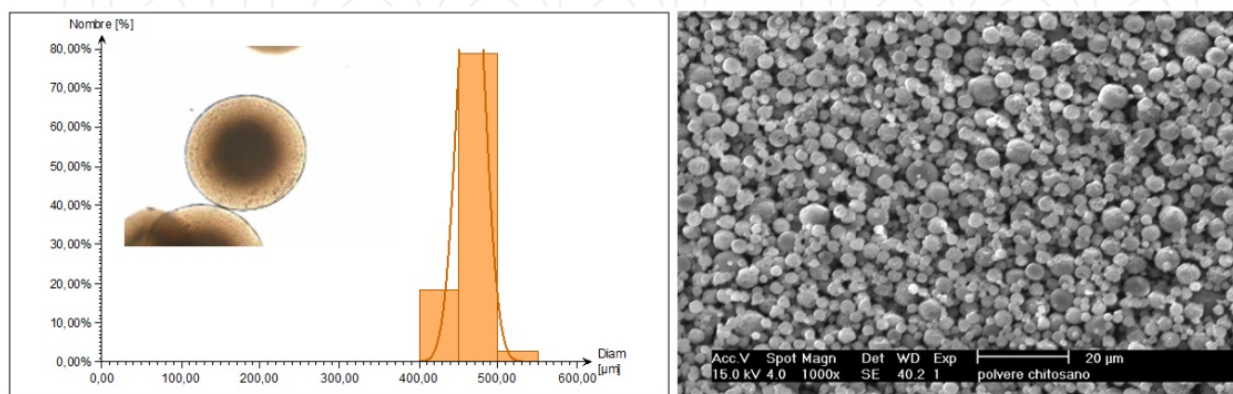
Figure 15. Chitosan microcapsules obtained by (left) TTP crosslinking using the IE-50R and size distribution of the particles, and (right) spray-dryer (TECNALIA).

Chitosan is biodegradable and biocompatible. Nevertheless, to be used in probiotic bacteria encapsulation it is necessary to consider the antibacterial activity of this polymer. Due the possibility of a negative impact in the viability of bacteria, and due that chitosan has a very good film-forming ability, chitosan is more used as external shell in capsules made with anionic polymers as alginate. This application of chitosan can improve the survival of the probiotic bacteria during storage and also in the gastrointestinal tract [67, 161, 162], and therefore, it is a good way of delivery of viable bacterial cells to the colon [67].

3.2.2. Thermal hydrogels

- **Gellan gum**

Gellan gum is a high molar mass anionic polyelectrolyte produced as an aerobic fermentation product by a pure culture of *Pseudomonas elodea* [163]. The chemical structure of gellan gum shows a tetrasaccharide repeating unit composed of one rhamnose, one glucoronic acid and two glucose units. It is possible to induce a thermo-reversible gelation upon cooling of gellan gum solutions and the gelation temperature will depend on the polymer concentration, ionic strength and type of counterions presents in the medium. The gels of gellan gums with low acyl content need the presence of divalent stabilizing cations [164].

Although gellan gum is able to generate gel-bead structure for microencapsulation, a disadvantage is that it is not used in this way for this purpose because of having a high gel-setting temperature (80-90°C for about 1 h) which results in heat injuries to the probiotic cells [129].

- **Xanthan**

Xanthan is a heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose units, two mannose units and one glucoronic acid unit. The polysaccharide is produced by fermentation of bacterium *Xanthomonas campestris* and posterior filtration or centrifugation. This polymer is soluble in cold water and hydrates rapidly. Even if xanthan is considered to be mainly non-gelling a mixture of both, xanthan and gellan gum has been used to encapsulate probiotic cells [19, 102] and contrary to alginate, the mixture presents high resistance towards acid conditions.

In contrary with alginate, mixture of xanthan-gellan is resistant to acidic conditions. Also, as opposed to from carrageenan which needs potassium ions for structural stabilization (it is harmful for the body in high concentrations), this gum can be stabilized with calcium ions [165, 166].

- **Carrageenan**

Carrageenans are a family of high molecular weight sulphated polysaccharides obtained from different species of marine red algae. The most frequently used is κ-carrageenan, opposite to λ- or τ-carrageenan. This polymer is largely used as thickening, gelling agent, texture enhancer or stabilizer on food, pharmaceutical and cosmetic formulations. His

primary structure is based on an alternating disaccharide repeating unit of α-(1,3)-δ-galactose-4-sulphate and η-(1,4)-3,6-anhydro-δ-galactose.

κ-carrageenan requires high temperatures (60-90 ºC) for dissolution, especially when applied at high concentrations such as 2-5%. However, this material used for encapsulating probiotics requires a temperature comprised between 40 and 50 ºC at which the cells are added to the polymer solution. It forms thermoreversible gels by cooling in presence of K$^+$ as stabilizing ions. The gelation with divalent cations as Ca^{2+} or Cu^{2+} is also possible, but not so often used and the thermal gelation is the most common method [167, 168].

The κ-carrageenan beads for probiotic encapsulation can be produced using several technologies described in the extrusion as well as emulsion techniques.

The encapsulation of probiotic cells in κ-carrageenan beads keeps the bacteria in a viable state [96] but the produced gels are brittle and are not able to withstand stresses [19].

- **Gelatin**

Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations and containing between 300 and 4,000 amino acid units. Gellatines generally have a characteristic primary structure determined by the parent collagen, because they are a irreversible hydrolysed form of collagen obtained from the skin, boiled crushed bones, connective tissues, organs and some intestines of animals. However they vary widely in their size and charge distribution and there are two types of gelatines depending on the treatment to obtain the gelatine: type-A gelatine is obtained from acid treated raw material and type-B gelatine is obtained from alkali treated one.

Gelatine is water-soluble, but the solutions have high viscosity and it forms a thermal hydrogel who melts to a liquid when heated and solidifies when cooled again. Gelatine gels exist over only a small temperature range, the upper limit being the melting point of the gel, which depends on gelatine grade and concentration (but is typically less than 35 °C) and the lower limit the freezing point at which ice crystallizes.

This material is useful to obtain beads using extrusion technologies or form a w/o emulsion by cooling, but to stabilize the gel the beads may need to be crosslinked using glutaraldehyde or salts of Chrome. In fact, it is largely used in complex coacervation technique combined with anionic polysaccharides such as arabic gum and others. The most important consideration is that both hydrocolloids have to be miscible at an appropriate pH to stabilize their charges and avoid the repulsion between similar charged groups.

3.2.3. Milk protein gel

Just like the gelatine, milk proteins are able to form gels in the suitable conditions. Proteins are chains of amino acid molecules connected by peptide bonds and there are may types of proteins due the high number of amino acids (22 units) and the different possibility of sequences. Among other proteins, milk proteins are very interesting as encapsulation

material by their physic-chemical properties. There are two major categories of mil protein that are broadly defined by their chemical composition and physical properties. The caseins are proline-rich, open-structured rheomorphic proteins which have distinct hydrophobic and hydrophilic parts and 95% of caseins are naturally self-assembled into casein micelles. Whey proteins primarily include α-lactalbumin, β-lactoglobulin, immunoglobulins, and serum albumin, but also numerous minor proteins, but whey proteins are globular ones.

Milk proteins are natural vehicles for probiotics cells and owing to their structural and physico-chemical properties, they can be used as a delivery system [169]. For example, the proteins have excellent gelation properties and this specificity has been recently exploited by Heidebach and collaborators [170, 171] to encapsulate probiotic cells. The results of these studies are promising and using milk proteins is an interesting way because of their biocompatibility [169].

3.2.4. Starch

Starch is a polysaccharide composed by α-D-glucose units linked by glycosidic bonds, produced by all green plants. It consist of two constitutionally identical but architecturally different molecules: amylose and amylopectin. The amylose is the linear and helical chains of glucose polymer, while the amylopectin is the highly branched chains. The content of each fraction depends of the starch origin, but in general it contains around 20-30% amylose and 70-80% amylopectin.
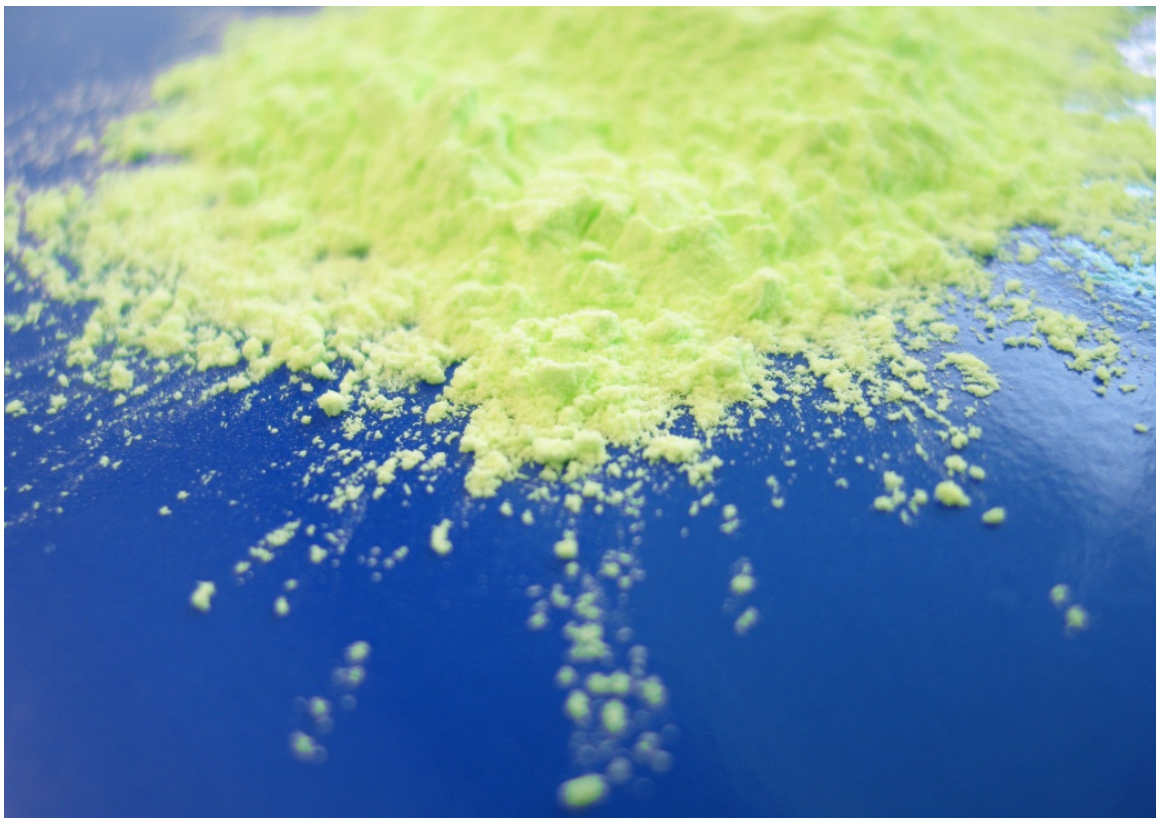
Figure 16. Coloured maltodextrin microcapsules obtained by spray-drying (TECNALIA).

As it is described in the previous section, the probiotic bacteria can be encapsulated by adhesion to starch granules, but usually the starch is chemical or physically modified for different applications, even encapsulation as maltodextrins or cyclodextrins commonly used in combination with the spray-drying technology (Figure 16), fluid bed granulation, for examples.

Starch granule is an ideal surface for the adherence of the probiotics cells and the resistant starch (the starch which is not digested by pancreatic enzymes in the small intestine) can reach the colon where it is fermented [172]. Therefore, the resistant starch provides good enteric delivery characteristic that is a better release of the bacterial cells in the large intestine. Moreover, by its prebiotic functionality, resistant starch can be used by probiotic bacteria in the large intestine [173, 174].

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