Review

Encapsulation of probiotics: insights into academic and industrial approaches

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Abstract: The natural inhabitants of the gastrointestinal tract play a key role in the maintenance of human health. Over the last century, the changes on the behavior of our modern society have impacted the diversity of this gut microbiome. Among the strategies to restore gut microbial homeostasis, the use of probiotics has received a lot of attention. Probiotics are living microorganisms that promote the host health when administered in adequate amounts. Its popularity increase in the marketplace in the last decade draws the interest of scientists in finding suitable methods capable of delivering adequate amounts of viable cells into the gastrointestinal tract. Encapsulation comes into the scene as an approach to enhance the cells survival during processing, storage and consumption.

This paper provides a comprehensive perspective of the probiotic field at present time focusing on the academia and industry scenarios in the past few years in terms of encapsulation technologies employed and research insights including patents. The analysis of the encapsulation technologies considering food processing costs and payload of viable bacteria reaching the gastrointestinal tract would result into successful market novelties. There is yet a necessity to bridge the gap between academia and industry.

Keywords: bacteria; microencapsulation; microparticles; probiotics; process

Abbreviations:
GIT: gastrointestinal tract, CFU: colony-forming unit, US FDA: United States Food and Drug Administration, MRS: Man, Rogosa and Sharpe

1. Introduction

Probiotics are living microorganisms that promote the host health when administered in adequate amounts [1]. Commonly the scientific community accepts a daily consumption of 10^8 to 10^9 CFU/g (Colony Forming Units per gram) of probiotic viable cells as being the minimum amount required to confer the desired benefits [2]. Thus US FDA recommends a minimum concentration of 10^6 CFU per ml or per g of probiotic viable cells right at consumption in the food product [3]. However, to date there are no uniform therapeutic dosing recommendations for probiotic intake. Doses range from 10^7 CFU /day to 10^{12} CFU/day, depending on the clinical endpoint, the probiotic genus, species or strain [4].

The Russian Nobel Prize Eli Metchnikoff was the first one to suggest the beneficial role of the gastrointestinal bacteria at the beginning of the twentieth-century. He based his hypothesis on the observation of the longevity of Bulgarian peasants and their frequent intake of fermented dairy products. Metchnikoff postulated that the lactic acid bacteria replaced the pathogenic microorganisms in the guts and the result was a decrease in certain illnesses [1]. At the same period of time, a French pediatrician called Henry Tissier observed the low numbers of a peculiar Y-shaped bacterium in the stools of infants facing diarrhea. He observed that healthy subjects had plenty of these 'bifid' microorganisms, which led him to suggest its administration to restore the microbiota of the ill children. Metchnikoff and Tissier were thus the pioneers of the probiotic field, although the term probiotic was not utilized until decades later [1].

Under natural circumstances humans should not need probiotic supplements with the exception of the public holding any type of special condition, e.g. long-term use of antibiotics. However, changes of the behaviors of our society challenge the inhabitants of the gut [5]. Sterile food consumption, increased hygienization, stress and antibiotics administration are some of the antagonists of our commensal microbiota, which can be potentially normalized by the ingestion of probiotics.

Today, a wide range of probiotic products, mainly dairy, is in the market, and more are yet to come, especially non-dairy novelties [6]. The low-cholesterol diets, lactose intolerance and milk protein allergy public, vegans and vegetarians are some of the future potential customers for these products. Herein some examples: table olives [7]; apple juice [8]; goat's milk ice cream [9]; green tea [10]; kefir [11]; dry apple snack [12]; orange, pineapple and cranberry juices [13]; pomegranate and cranberry juices [14]; carrot and watermelon juice [15]; fresh apple wedges [16,17]; chocolate [18] and cereals [19,20]. Some probiotic products have the drug status and are prescribed as anti-diarrheic, e.g., Lacteol®, Lactobiane® and Bacilor®. The global economic scenario of probiotics is very encouraging. Probiotic ingredients and supplements reached approximately $23.1 billion in 2012; and the growth forecast is in the $36.7 billion range to 2018, with a compound annual growth rate of 6.2% in the next four years [21].

The maintenance of the cell viability in probiotic-containing products is still considerably challenging. The microorganisms must survive during the industrial processing, which is often detrimental to the cell viability in the final product. As these products are intended for oral use, the remaining viable cells undergo further stresses during the gastrointestinal passage due to changes in pH or the contact with bile salts, which might disrupt the membrane of microorganisms. As a consequence, additional loss in cell viability is expected before they even reach their target.

In this context, the encapsulation of probiotics could provide the required protection to the administrated bacterial cells and thus, increase the delivered amount. Piano et al. demonstrated through several clinical studies that microencapsulated probiotics were significantly more effective to deliver viable cells to the colon than the non-encapsulated counterparts [22,23]. The authors reported that subjects who received microencapsulated cells showed a similar intestinal colonization profile with respect to the subjects who received 5-time higher amounts of non-encapsulated cells.

In order to successfully encapsulate viable cells into foodstuff, it is of paramount importance to preserve the bacterial viability in all of the manipulation procedures along with choosing accordingly the encapsulation materials, which must be food compatible [24,25]. Additionally, the microcapsules must maintain the cell viability during their storage alone or when added to food carriers. For instance, high relative humidity in the final product will favor the oxidative process of the matrix and therefore be detrimental to the cells [26]. Teixeira et al. reported an optimal relative humidity of 7–11% for bacterial survival [27].

Although valuable and encouraging research has been published over the years and commercialization of products is being made, the information exchange between industry and academia should noticeably intensify. Encapsulation of probiotics and food delivery systems are a rapid growing research theme. This paper aims to give an overview of the vast probiotic field on what is being done in the academia and industry scenarios in the past few years, in terms of technologies employed and research insights. For such, mostly recent and/or other important published work including patents is the focus of this review.

2. Microcapsules Morphology and Encapsulating Agents

Scientists assess a multitude of technological approaches for obtaining microcapsules. The final morphology obtained and diameter will depend on technological process and materials implied during the microcapsule formation.

Several materials have been extensively researched up to date for probiotic encapsulation purposes. Some are very popular with a long reputation of use such as alginate. Alginate forms matrix microspheres that can be obtained by extrusion. When a hydrophobic coating is applied over the alginate matrix, the result is a shell-matrix microcapsule, and if the matrix is a bulk, then the microcapsule is a shell-core-type (Figure 1) [28,29]. Materials used for probiotic encapsulation were previously reported in several reviews [30–33].

Table 1 resumes pros and cons for each technology correlating them with current encapsulation agents and final capsules sizes. To access information on cell immobilization procedures and more details on materials used for microencapsulation refer to Poncelet et al. [34] and Oxley et al. [28], respectively. In terms of technological processes spray drying, spray cooling, spray coating, emulsification, liposomes, coacervation and extrusion are addressed in more detail. It is important to keep in mind that frequently a combination of these technologies is applied in order to obtain the intended encapsulated material.
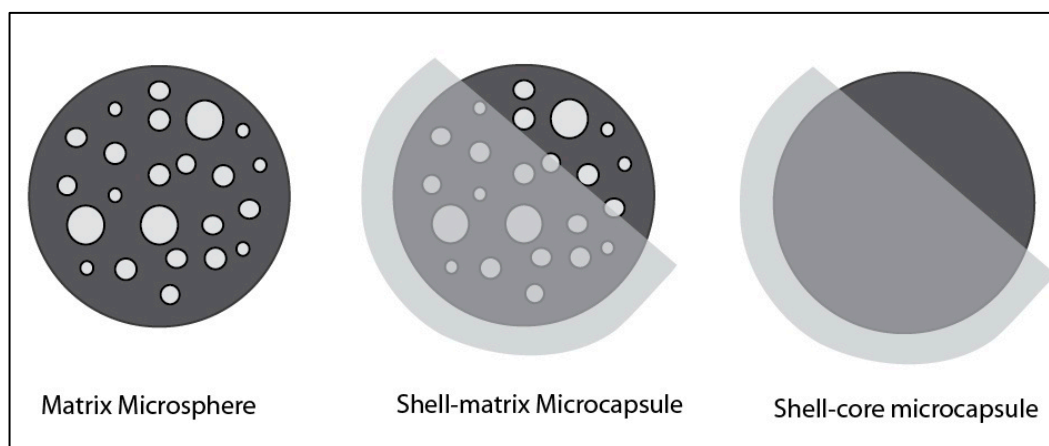
Figure 1. Fundamental components of an encapsulated particle: Matrix microsphere, shell-matrix and shell-core microcapsules.

Table 1. Correlation of encapsulation technologies with capsules sizes, most current encapsulation agents, pros and cons.

	Usual Capsules sizes	Most current encapsulation agents	Pros	Cons
Spray drying	5 to 150 µm [35]	Inulin [36,37], acacia gum [38], locust bean gum [39], starch [8], soy protein [38], whey protein [37,40,41,43] skim milk [36], chitosan [42] [43], alginate [43]	1. Mass production, 2. Continuous process, 3. Material monodispersity, 4. Well established in the food industry for other applications, 5. Inexpensive.	1. Loss of cell viability due to high temperatures, 2. Mostly used with aqueous suspensions, i.e. shell material should be soluble in water [28].
Spray cooling	20 to 200 µm [35]	Cocoa butter [44], Palm oil [45], Palm kernel oil [45]	1. Mass production, 2. Continuous process, 3. The least expensive encapsulation technique [46]; 4. Mild temperatures setup.	1. Lower load (10-20%) when compared to spray drying (5-50%) [35]. 2. Encapsulated ingredient may be on the surface and in contact with environment (matrix spheres) [46], 3. Difficulty to delay the release of water-soluble ingredient over 30min [46].
Freeze drying	-	Starch [47], soy protein [20,48], casein [48], whey protein [48], skim milk [48]	1. Excellet final dried material suitable for most food applications [28], 2. Suitable for sensitive materials as probiotics.	1. High costs, 2. Cell damage with eventual crystal formation, if not done correctly [28], 3. Eventual need of cryoprotectants.
Spray coating	5 µm to 1 mm [28]	Shellac [39], casein [49]	1. Control release with addition of different coatings, 2. Increase in storage stability.	1. May introduce forces that can damage the cells [28], 2. Although temperature is lower, the exposure could be longer along with oxygen exposure [28].
Emulsifi-cation	200 nm to 1 mm [28]	Inulin [50], K-carrageenan [51], alginate [52]	1. Easy technique, 2. Usual high survival rate of bacteria.	1. Costly to scale-up, 2. Polydispersity of material, 3. Shape variation of material, 4. Prolonged shear forces may cause

				damage to cells.
Liposomes	few nm to few µm [28]	Phospholipids [28]	1.Improve taste issues (e.g. flavor encapsulation), 2.Provide good protection to sensitive agents [28], 3. Can carry hydrophobic and hydrophilic molecules in the same cargo, 4.Mucoadhesive [28].	1.Most costly and complex technique among the ones described in this review [28], 2. It uses organic solvents [28], 3.Formulations are kept in dilution aqueous solution, 4. Stability issues at high and room temperature as well as low pH [28].
Coacervation	1µm to 1 mm [28]	Pectin [49], gelatin [53]	1.High payloads (up to 99%) [46], 2.Mild preparation conditions, 3.High shell integrity.	1.Expensive technology, 2.Usual use of non-food grade crosslinkers or enzymes, which are not time efficient up to today [46], 3.Natural small differences in the structure of the polysaccharides influence the final capsule structure (quality control challenge).
Extrusion	100 µm to 3 mm [28]	Locus bean gum [39], pectin [14], chicory [54], sugarbeet [54], whey protein [55], gelan gum [56], xanthan gum [56], gelatin [14], chitosan [14], K-carregeenan [51], alginate [14,54,52,57,58]	1.Monodispersity, 2.Scale-up potential, 3.Mild conditions, 4.Continous process	1.Frequently other technologies are further used in order to obtain a final dry material, 2.Larger capsules.

2.1. Spray drying

This atomization technology is the most widely used encapsulation technique in the food industry due to its continuous production feasibility and tailor-made characteristics depending on feed and encapsulating agent [28]. The configuration of a basic spray dryer consists of a drying chamber, which receives a liquid spray, and it is rapidly evaporated as soon as it encounters the hot air flow (Figure 2). The liquid is broken into fine dried droplets. The air flow may be concurrent, such as in the most food industry spray-driers allowing a rapid drying [59], but also mixed or countercurrent [60]. Industrial equipment has evaporating rates in the range of 0.1 to 12 t/h.

Spray driers are used frequently to entrap oils, aroma, enzymes and pharmaceutical drugs. A large amount of research has been put into encapsulating probiotic bacteria in a laboratory scale; however, final cell viability in food carriers can still be an issue for industrial application due to temperature, osmotic extremes and water activity (a_w).
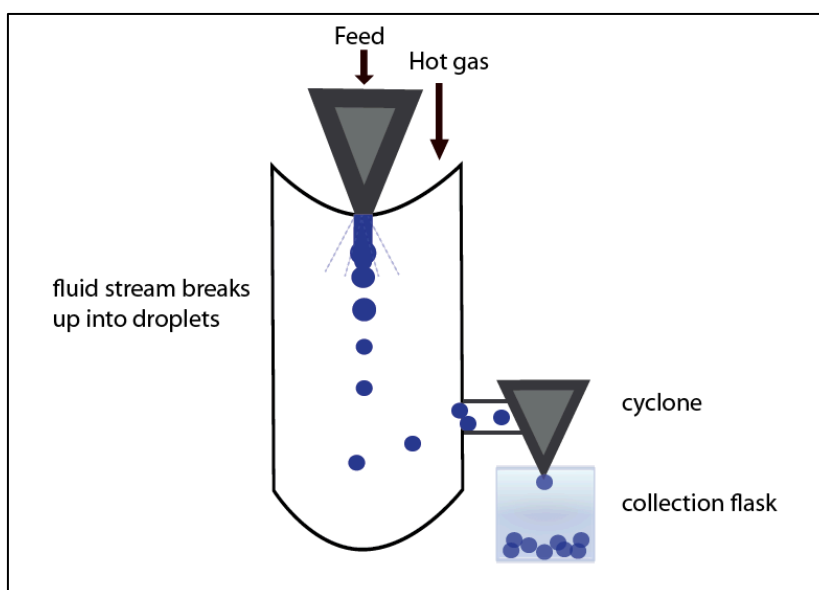
Figure 2. Basic schema of a concurrent spray dryer.

Academia: Considerable amount of spray-dried probiotic papers are published every year. Herein relatively recent work has been summarized. Yonekura et al. used sodium alginate, chitosan and hydroxypropyl methylcellulose (HPMC) as co-encapsulants in the spray drying of *L. acidophilus.* The authors report that chitosan reduces viable counts after spray drying whereas HPMC does not. However, the former enhances storage stability and higher viable counts were achieved at a 35-day storage period [43]. The study points out the importance of evaluating the viability during storage and not only after the encapsulation procedure, since for industrial purposes, the cells protection is essential during the storage.

Castro-Cislaghi et al. encapsulated *B. animalis* ssp *lactis* in milk whey. The viability of the capsules remained high and constant (> 9 log CFU/g) for 12 weeks when stored at 4 °C [41]. However, the cells were not protected against the bile salts, which is fundamental for the final application. Nevertheless, as also reported by Rodriguez-Huezo, the observed 'flat ball effect' phenomenon provides the capsules with a certain mechanical robustness to mechanical fractures [61].

Behboudi-Jobbehdar et al. stabilized *L. acidophilus* in a maltodextrin/whey protein concentrate/ D-glucose carrier. The increase in the survival rate of the cells from 2.5 to 84% when the outlet temperature was reduced from 91.5 to 60 °C was a meaningful finding [40]. In addition, the authors concluded that a more elevated flow rate typically reduces cell membrane damage.

Ying et al. encapsulated *L. rhamnosus* in whey protein and/or resistant starch matrices followed by a probiotic survival investigation over a 5-week storage period at 4 or 25 °C in citrate buffer (pH of 3.5) or in apple juice. The reduction in cell numbers was greater for corresponding formulations in citrate buffer than in the juice, where the pH also slightly decrease over time and cell density increased [37]. *L. rhamnosus* is known to be an acid-resistant strain, and this work nicely indicates that the strain can even better withstand acidic conditions when metabolizing substrates are available in the media.

Corcoran et al. spray-dried *L. rhamnosus* in lag, early log and stationary phases and compared the strain viability independently of the type of feed carrier used. The order of survivability is

stationary, early log and lag with 50%, 14% and 2% respectively [62]. A unquestionably important parameter that has to be considered at the beginning of the studies.

Finally, in order to aid the cells resist the dehydration process, osmoprotectants such as trehalose [63], non-fat milk solids or adonitol [64] are added in the encapsulation systems. These compounds influence the changes in the osmolality of the surroundings, the cell cytoplasm, and the tension in the cell envelope [64].

Patents and Industry: The development of spray-dried probiotic bacteria has been limited in large commercial scale to the best of our knowledge. Despite the high production rates and relatively low operating costs, *i.e.* 4 to 7 times cheaper than freeze drying [38], most of the probiotic strains do not survive in large quantities after the heat stress and dehydration. The high temperatures result in high mortality and inactivation of microorganisms damaging cytoplasmatic membrane, cell wall, ribosomes and DNA [59,65]. Another industrial limitation is the shell material, which needs to be soluble in water at an acceptable level due to usual water aqueous formulations [46].

Table 2. Overview of key considerations when considering spray-drying technology for probiotic encapsulation. Source: Information adapted from [38].

	Remarks	Effect
Type of bacteria and stress tolerance	It is strain-dependent.	Stress tolerance is an intrinsic physiological function.
Carrier	Carriers are typically proteins and/or carbohydrates.	They help bacteria reach their site of action with certain viability or completely viable.
Drying temperature	The sensitiveness is strain-dependent.	Mortality increases with drying temperature and it is accentuated typically above 51°C [67]. Survival rate was observed to be inversely proportional to outlet temperature [66].
Time of exposure to heat	Mortality is proportional to the time of exposure.	Heating time should be as short as possible.
Osmotic, oxidative, mechanical stresses	It is strain-dependent.	Additives such as antioxidants or osmoprotectants can be included. Pre-stressed bacteria before drying can improve survival ('survival of the fittest') [66].
Glass transition	Glassy state for food powder.	Food powder should be in the glassy state to minimize molecular mobility and avoid further detrimental processes [68].
Encapsulation, coating	Barriers against the harsh environment.	Coating enhances stability, especially during the gastrointestinal passage.
Water activity (a_w), moisture content	In order to ensure stability, a_w should be below 0.25. Moisture content should be below 5% [66]	a_w values between 0.11 and 0.23 prevented cell death during storage and higher values were related to the accelerated mortality of *L. delbrueckii* [27]
Storage conditions	Microcapsules must be protected from heat, oxygen and moisture. Proper packing material must be selected accordingly.	Survival is inversely related to storage temperature. Decreasing order of spray-dried bifidobacteria survival: vaccum laminated pouch > glass bottle > PET bottle [69]. Vaccum storage was proven to be better than nitrogen or air, respectively [70].

Stable spray dried culture starters are attractive to the fermented probiotic business as they could be added directly into finished food, suppressing the need of liquid starter stocks and resulting in less storage costs [66]. Table 2 exemplifies some aspects that should be considered to potentially improve the chances of a successful spray drying encapsulation [38].

2.2. Spray cooling (or spray chilling)

The active ingredient is dispersed in a molten material, usually fats, serving as the carrier. The cold air injected into the chamber enables the solidification of the microcapsules [71]. Customarily spray cooling refers to a matrix encapsulation (Figure 1) due to the aggregation of the active ingredients, which can also be found on the surface of the microsphere and therefore are readily released [46].

The advantages of this technique are: (i) the least expensive with scale-up potential for probiotics [44,46]. (ii) application of lower temperatures enabling its use with thermosensitive microorganisms, (iii) capsules are generally insoluble in water due to the use of wax and oil as carriers. Their solubility will depend on the hydrophilic and lipophilic characteristics of the carrier [35]. On the other hand, few eventual hurdles are: (i) displacement of the core material during storage due to the solidification and crystallization process of lipids [44], (ii) hydrophobic characteristic of the material can be an issue for certain applications, (iii) difficulty to obtained a delayed release of a water soluble ingredient over 30 minutes when the food carrier has high water activity [46]. Enzymes, flavors, minerals and proteins have been encapsulated using this technology [71].

Academia: This technique is less addressed in the probiotic field as far as our knowledge concerning scientific papers available. Pedroso et al. microencapsulated *B. animalis* ssp *lactis* and *L. acidophilus* with cocoa butter. The emulsion was formed using a high sheer device rotating at 9500 rpm for 60 s with subsequent atomization in a 10 °C chamber. Surprisingly the process conditions did not impact negatively the cell viability. *B. animalis ssp lactis* free and encapsulated showed the same resistance to simulated gastrointestinal fluids, however free cells were unstable during storage, and 72% of the encapsulated cells remained viable after 90 days of storage at 7 °C. In the case of *L. acidophilus* encapsulation increased its viability in simulated gastrointestinal fluid, but only 20% of the cells remain viable in the above storage conditions [44]. When considering an industrial application, this study confirms the relevance in choosing adapted strains in respect to the finished product conditions.

Okuro et al. produced solid lipid microparticles with inulin or polydextrose containing *L. acidophilus*. The polydextrose formulation presented the best cell viability with 6.9 or 6.8 log CFU/g at relative humidity of 11% after 120 days of storage at −18 °C or 7 °C, respectively [45]. This symbiotic system is an interesting deliver vehicle containing the minimum concentration of viable cells required by the US FDA.

Patents and Industry: A shell-core type of microcapsule has slower release kinetics due to its outer layer, often hydrophobic, which can be composed of single (Figure 1) or multiple shells. The advantage of having shells lies on the ability to tune the wall thickness and customize the microcapsule to the desired purpose. Such approach should be taken with careful judgment considering the possible substantial inert material inclusion into the product matrix. Detrimental

organoleptic effects and the impact in the nutritional-value of the product are eventual industrial hurdles.

Rutherford et al. successfully developed a process of spray cooling using a rotary disc for the animal feed field. The microspheres of fatty acid matrix (e.g. stearic acid) contain a plurality of individual microorganisms, such as *Enterococcus faecium*, *Lactobacilli* or yeast. The microspheres can be stored for a period of 3 to 6 months and cell viability is maintained even if there is exposure to some moisture or antibiotics [72]. DuPont Pioneer owns the patent.

The expanding scenario of probiotics gains overtime more attention in the animal feed industry as well where the rules for antibiotics administration become stricter and the intention of enhancing animal health and productivity is a reality [73]. Lallemand Health Solutions commercializes Bactocell®, a product based on viable *Pediococcus acidilactici* an additive that reduces bone deformation in fishes [74]. Companies such as Performance Probiotics are entire dedicated to the cattle feed business.

2.3. Freeze drying (or lyophilisation)

In a typical freeze-drying process, the liquid material containing the bacteria is first frozen with further decrease of the chamber pressure enabling the frozen water to sublimate. It is an energetic costly procedure, however there are at least two main advantages: (i) oxidation is avoided and, (ii) the drying step is less damaging to the cells when compared to the high drying temperatures. An eventual concern associated with this technique is the formation of ice crystals during the slow freezing, which may lead into significant cell damage if not handled correctly [63]. In order to attenuate cell loss, anti-freeze agents, known as cryoprotectants, are added to lower the freezing point of water and consequently its vapor pressure [28]. Ideal cryoprotectants should be food grade, permeate through the cell wall and contain non-toxic solutes. The synergistic effect of trehalose with sugar alcohols, glycerol or certain proteins allows a superior stabilization of the bacteria mixture during storage at long periods of time [75].

Academia: The technique of freeze drying is often utilized to preserve sensitive biological substances and to date is the best process to dry bacteria due to the maintenance of the cells viability during storage [3]. Nguyen at al. exposed *B. bifidum,* harvested in stationary phase, to 42°C for 100 s and 300 s, and verified the strain increased its freeze drying resistance. The authors observed changes in the zeta potential due to higher concentration of exopolysaccharides (EPS) secreted by the bacteria after the heat shock [76]. EPS are known to induce stress resistance and in a way, stressing the cells prior to freeze drying, increase their membranes robustness and as a consequence their survivability.

Basholli-Salihu et al. studied the effect of cellobiose, lactose, trehalose and sucrose, as cryoprotectants in the survivability of *B. infantis* in milk (pH 6.7) and grape juice (pH 3.4) at 4 °C after freeze drying. All four protectants responded in a similar way during a 2-week storage in milk with about no changes in viable counts in respect to time zero. However, cellobiose (5% and 10%) and trehalose (10%) lost only one log after a 4-weeks storage in grape juice compared to time zero [77]. If the storage of cells in low pH is of interest, then trehalose and cellobiose seem to be better choices of cryoprotectants. Conrad et al. verified 14 years earlier the potential use of a trehalose-borate system as cryoprotectant.[63]. The study suggests that borate can enhance this

protective ability of trehalose. Nevertheless, borax, the borate source of the above study, is an illegal food additive in several countries.

Hugo et al. confirmed the formation of stable cold-set gels with viable *L. delbrueckii* ssp *lactis* when adding CaCl$_2$ to high pressure-treated and freeze-dried soybean protein isolates. This formulation could be interesting for the non-dairy probiotic market.

Ananta et al. reported sucrose as being the most effective cryoprotectant over betaine for *B. animalis ssp lactis* and *L. rhamnosus*. Starch was also investigated as a carrier for its exceptional touch in the final product. A dried product containing starch is granular and light in color allowing its use in different food matrices. Nevertheless, its addition did not improve storage stability [66].

Patents and Industry: Oftentimes the high cost of this technique is highlighted as a drawback. However, when considering the bacterial loss in the cost analysis of different drying methods, freeze drying seems a competitive approach [28]. At present day the industry uses this technique to dry components for value-added products, e.g. enzymes, probiotic pills for the reestablishment of the gut flora and bacterial strains used in research. Traditionally cheesemakers purchase their freeze-dried starter cultures from specialized suppliers [70].

2.4. Spray coating

Spray coating consists of forming a uniform layer onto solid particles. It is utilized with a vast variety of shell materials such as polysaccharides, proteins, fats, yeast cell extract, or even complex formulations. This versatility translates into potential controlled release features [46]. Despite the three different air inlet positions, the basic functioning is the same. Powdered probiotic bacteria are kept in motion in the chamber and a coating is sprayed over them. The final material will have different characteristics depending on the applied spray coating configuration [78]. Fluidized bed drying is a type of spray coating invented and patented in 1963 by Würster.

Academia: Several nutraceuticals, such as supplements and pills, are encapsulated using spray coating. Schell et al. developed a microencapsulation system in a two-step process for *L. reuteri* using sweet whey protein and dietary shellac. The bacteria slurry was first top-spray with sweet whey along with trehalose, maltodextrin or sorbitol and a posterior shellac coating. Trehalose and sorbitol gave the highest viability of encapsulated bacteria. In this study, shellac coated bacteria survived best at pH 1.5 to 4.5 compared to only whey or free cells [79]. The uncommon use of shellac in the probiotic encapsulation turns the study an innovative approach. This resin is secreted by *Laccifer Lacca, a* female insect and it is FDA-approved to coat candies and fruits.

Semyonov et al. produced microcapsules containing *L. paracasei* using a Würster coater system with a bottom-spraying atomizer. The authors investigated the use of different coatings. Hydrogenated vegetable oil provided excellent moisture protection with 99% of cell survivability and ethylcellulose conferred protection during the gastrointestinal passage. The inlet air temperature was the most detrimental parameter with a 250-fold decrease in survival rate when a 15 °C temperature increment was applied [80].

Bensch et al. investigated the effects of fluidized bed drying on the viability of *L. plantarum* ssp. *plantarum* via flow cytometric and plate count methods. The difference shown between both methodologies is suggested by the authors as an occurrence of viable but nonculturable cells during storage [81]. Flow cytometry has potential for a rapid routine in-process industrial control while plate count is a laborious and time-consuming technique.

Champagne et al. evaluated various homogenization, rehydration and plating practices of freeze-dried *L. rhamnosus* and *B. longum,* which were spray coated with fractionated palm oil and palm kernel oil. This study points out that *L. rhamnosus* has a better survival rate to freeze drying than *B. logum* and spray-coated cultures rehydrated slower and only partially [82]. The rehydration may be a crucial factor when the encapsulated material is blended with a given product.

Patents and Industry: Fluidized bed drying is commonly used in the food industry. This technology allows the use of milder temperatures compared to spray drying due to optimal heat and mass transport resulting in higher cell survival [79].

Wu et al. coated lactic acid bacteria with high laurate canola oil (HLCO) holding a low melting point. Not only higher yields of encapsulated bacteria were observed when compared to the use of hydrogenated vegetable oils, but the use of HLCO lower temperature reduces smearing, caking and doughing up [83]. As a consequence, a product containing no cracks with a free flowing profile is homogeneously produced.

Ubbink et al. invented a probiotic delivery system where 0.02 cm^3 compacted pellets containing probiotics and further fillers improved the stability of the microorganisms when applied in semi-dry and/or humid particulate foodstuffs [84]. *Enterococcus faecium* has about 80% of viable cells recovery after a 30 days storage period at 30°C in 70% relative humidity. The patent belongs to Nestlé SA.

Lallemand Health Solutions has developed an encapsulation technology named Probiocap®. The molten hydrophobic coating, a mixture of at least one fatty acid and/or wax, is injected in a rotational vessel containing agglomerates of living dehydrated probiotics [85]. One of the advantages of the process includes suitability with a wide range of coating materials, which can have a melting point greater than the temperature of the human body avoiding an early stage disintegration of the capsules. The same company developed Star®, a water-based enteric-coating with excellent barrier protection that allows manufactures to add a lower concentration of microorganisms per capsule.

2.5. Emulsification

An emulsion is a mixture of two or more immiscible liquids where one liquid (the dispersed phase) is dispersed in the other (the continuous phase) under the stabilizing effect of surfactants. The most common emulsions are water-in-oil (w/o), oil-in-water (o/w) and water-in-oil-in-water (w/o/w).

Emulsion-based processes have a drawback in regard to the difficulty in controlling the size distribution of the droplets. In order to produce monodisperse droplets, there are two emerging technologies: membrane emulsfication and microfluidic systems. The use of low sheer stresses with these techniques makes them suitable for sensitive compounds.

Academia: Emulsion-based techniques are on the radar of many researchers. Mantzouridou et al. studied inulin-based emulsions. *L. paracasei* ssp *paracasei* was either suspended in the aqueous phase or entrapped in corn oil droplets of three emulsion systems containing egg yolk, gum arabic/xanthan or whey protein isolate. The free cell control in the aqueous phase and stabilized with egg yolk had the highest cell viability after a 4-week refrigerated storage when compared to the cells imprisoned in oil droplets. Inulin enhances the stability of the emulsion by providing stronger interactions with the oil droplets, and it seems to support probiotic culture growth [50].

Amine et al. studied the survival of *B. longum* in cheddar cheese in terms of freezing tolerance, storage in a frozen state, cheese manufacturing and stimulated gastrointestinal conditions. The

technologies of co-axial airflow extrusion and emulsion along with the use of native and palmitoylated alginate as encapsulating agents were exploited. A 30-days storage at – 80 °C showed similar viable counts for free cells and both encapsulation systems when utilizing either alginate [52]. The alginate confers protection mostly during gastrointestinal passage and storage of the cheese.

Dianawati et al. prepared fifteen different emulsion systems to evaluate the survival of *B. longum* after freezing steps. Sodium caseinate, whey protein concentrate, sodium caseinate:whey protein concentrate, skim milk, soy protein were combined with glycerol, mannitol or maltodextrin. Overall milk proteins and sugar alcohols were more effective than soy protein isolate and maltodextrin [48]. One explanation may be due to the easier interaction of the hydroxyl groups of sugar alcohols with the polar sites of phospholipid bilayers composing the cell membranes.

Patents and Industry: Herein some interesting patents based on emulsion techniques. Beck et al. elaborated a w/o/w edible emulsion containing probiotic bacteria in the internal water phase. The emulsion has an acceptable level of viable cells after 3 weeks of storage at 8 °C [86]. A particular advantage is that it holds no volume restrictions in respect to the concentration of probiotics that can be added to the internal phase. The invention relates to an emulsion for salad dressings and belongs to Unilever.

Vos et al. created a method to produce stable w/o/w emulsion useful to probiotics. It holds the advantage of masking the flavors of the aqueous components of the inner water phase and maintaining their stability for longer [87]. The emulsion components are common in the academia studies such as sunflower oil and skimmed milk powder except for admul (an emulsifier) and hipotral 35 (a whey protein source).

Mazer et al. patented a method for producing a stable emulsion that is further added into a powdered nutritional product designed to infants, children or adults. Any acidophilic and/or bifidobacteria can be incorporated into the method [88]. Abbott GmbH holds the rights on it.

The size distribution of emulsion droplets produced by simple high sheer methods is often seen as a drawback. Nowadays it can be solved by the microfluidic technology. There are at least two companies that use the microfluidic systems to manufacture monodisperse droplets. Nanomi manufactures injectable pharmaceutical products and Micropore® Technologies transforms their knowledge into application in different fields, such as nutraceuticals delivery or chromatography. The microfluidic technique has tremendous size distribution homogeneity; however, the high cost of final product is still a hurdle for the production of ordinary foodstuff.

2.6. Liposomes

Liposomes are spherical bilayers composed of phospholipids similar to cellular membranes. Hydrophilic molecules are entrapped in the water-soluble interior of the liposome, and hydrophobic in the oil-like portion. A potential use of liposomes lies on using one single cargo to delivery hydrophilic and hydrophobic active ingredients. The large unilamellar vesicles (LUV) are the most pertinent for the food industry due to their high encapsulation efficiency, straightforward production methods and over time better stability [46]. Main drawbacks of such technique are upscaling costs and often the formulations are kept in rather dilute aqueous suspensions, which translate into additional costs [46].

Academia: Liposomes are used in a diversity of products in the beverage, cheese industries and in the elaboration of vaccines. Enzymes, small molecules and antimicrobials are some of the

examples in which this technique is used [28]. To the best of our knowledge, little has been done currently in the probiotic field. Nevertheless, giant liposomes having a diameter greater than 1 μm are of particular interest in the encapsulation of bacterial cells, which possess a size ranging between 1 and 4 μm. Their stabilization may be an issue along with over time oxidation, aggregation and fusion.

Patents and Industry: There are still several issues to be addressed in order for liposomes to be successfully utilized in the probiotic industry. Chemical degradation, stability and oxidation are main downsides. The use of liposomes in value-added products is however current and well established in the pharmaceutical industry. For the beverage industry, one smart development to package liposomes and avoid the lipid degradation is by freeze drying it. Curcusome®, a freeze-dried liposomal formulation of curcumin/quercetin/piperine can be kept in release caps. Since liposomes are not stable at low pH or high temperatures, often parameters of the beverage industry, the liposomal powder is released from the cap into the beverage right at consumption. Innovations as such can easier bridge the liposomes research to probiotics.

Frenken et al. published a patent for treating, reducing or preventing diarrhea and treating rotavirus infection. The food or pharmaceutical preparation has antibodies, antibody fragments and probiotics. The eventual invention may be an additive in margarine, ice-cream, dressing or even beverages [89]. Unilever owns the rights.

Gregoriadis et al. patented a method for the formation of liposomes with a vesicle diameter smaller than 50 μm containing one or more entrapped *Bacillus subtilis*. After the formation of it, the liposome is freeze dried. The cell viability maintains stable after the dehydration/rehydration processes [90].

2.7. Coacervation

It is a well-developed and widely studied technique applied with several hydrocolloid systems such as gelatin/acacia gum, carrageenan, chitosan, soy protein and gelatin/carboxymethylcellulose. Coacervation can achieve rather high payloads, on the other hand the process cost is expensive [28] and frequently crosslinking substances are involved, such as glutaraldehyde or enzymes [46]. One (simple coacervation) or more (complex coacervation) hydrocolloids are dispersed in an aqueous solution with an active substance. With a pH change, the opposite charged colloids link together forming a layer around the active substance.

Academia: This technique is more of a matrix type (Figure 1), which means the bacteria might be present on the surface of the microsphere. Gerez et al. encapsulated *L. rhamnosus* combining ionotropic gelation using pectin and pectin-whey protein and further complex coacervation of whey-protein forming a coating without involving the use of organic solvents. Electrostatic complexes are formed below the isoelectric point of the whey protein (pH 5.4) where mainly positively charged NH_3^+ of the whey protein is attracted by the carboxylate groups of the pectin. The authors observed an effective protection of *L. rhamnosus* with the pectin-whey protein formulation resulting in a survival rate of 10^7 CFU/ml in pH 2 [91].

Shoji et al. microencapsulated *L. acidophilus* by complex coacervation employing pectin and casein followed by spray drying and further addition it to buffalo milk yogurt. The bacteria survived in 10^7 CFU/g in buffalo milk presenting a shelf life of around 120 days at 7 °C. However, adequate amounts did not survive the simulated pH conditions of the stomach [49].

Patents and Industry: The complex coacervation has been used widely to encapsulate flavors and unsaturated fatty acids in the industry with the goal of enhancing their shelf life. Bazo Agüeros et al. developed a method for the encapsulating of probiotic bacteria producing microparticles with a matrix composed of casein and chitosan. The capsules size is controllable with diameters below 100 μm [92]. This patent is an example of eventual industrial feasibility of the coacervation method for the encapsulation of probiotics. The inventors were able to obtain capsules with sizes in agreement with the food industry requirements.

2.8. Extrusion

There is a considerable amount of extrusion methods, such as dripping by gravity, electrostatic potential, vibration for jet break-up, JetCutter, and others [59]. This technology holds the basic concept of dispersing the bacteria in a solution that goes through a dripping nozzle and the droplets harden simultaneously by gelation when contacting with a second solution.

Electrospraying is a microencapsulation emerging technology and relies on the application of high potential electrical field to obtain spheres. The polymer solution is extruded from a capillary where two major electrostatic forces are acting: the repulsion of like charges and the Coulombic forces of the external electrical field. At the tip of the needle, the droplet is distorted in a conical shape, and once the electrostatic forces counteract the surface tension of the bead, a charged droplet is ejected from the tip of the cone [93]. Vibration for jet break-up and JetCutter will break the stream of liquid with vibration and a rotating cutting tool, respectively. The formation of uniform beads and the use of mild temperatures are an advantage of the extrusion techniques.

Academia: Extrusion is largely applied in research and sometimes considered as a laborious technique. However, with the development of different technologies, which go beyond the batch processes, industrial applications should be on the rise. Borges et al. evaluated the effect of microencapsulation on the viability of *L.casei*, *L. paracasei*, *L. acidophilus* and *B. animalis* in an alginate matrix. The effects of L-cysteine addition, exposure to pH of 3, NaCl (25% w/v) and temperatures of 55 °C and 60 °C were investigated. Results concluded that survival is strain-dependent. Free and encapsulated *L. acidophilus* demonstrated the highest survival rates when lethal conditions of temperature and pH were applied [94]. The authors discuss the final bead size and its protection role. The requirement of the food industry to avoid organoleptic drawbacks in a given product is of 100 μm and in fact humans can detect particles of sizes small as 25 μm [95]. However, smaller sizes confer less protection. Finally, the balance between final size and intended protection is debatable and it will ultimately depend on the characteristics of the food carrier.

Nualkaekul et al. compared alginate and pectin beads for improving the survival of *L. plantarum* and *B. longum* during storage in pomegranate and cranberry juices. The influence of various coating materials including chitosan, gelatin and glucomannan on cell survival was assessed. The double gelatin coated pectin beads coffered the highest protection in the juices. The effect may be attributed to the formation of a dense polyelectrolyte complex, which eventually increased the buffering effect of the beads. For instance, the coating protected the beads against the penetration of gallic acid [14]. It is a very important result since there are large numbers of studies using the alginate/chitosan system, yet chitosan is allowed in the pharmaceutical but not in the food industry at present time as far as our knowledge.

Khan et al. entrapped *B. adolescentis* in capsules composed of biopolymer mixtures of chickpea, faba, lentil or pea protein isolates with alginate. Shelf life studies with plain yogurt as a model showed a reduction of 3 $\log_{10}$ CFU/ml for entrapped cells with all capsules designs in a period of 7 days whereas free cells had 8 $\log_{10}$ CFU/ml reduction [58]. The use of legume proteins along with alginate is novel and this approach opens up the ingredient market to non-dairy applications and, consequently, to a different customer niche.

Laelorspoen et al. investigated the use of electrospraying in a zein-alginate core-shell system. When increasing the voltage from 4 to 10 kV, the capsules size decreased from 543 to 259 μm and the viable counts of *L. acidophilus* suffer only a minor change from 8.85 to 8.31 log CFU/ml, respectively [96]. The electrospraying technique for bacteria encapsulation shows therefore a great potential. The mild conditions used along with scale-up possibilities are indeed positive aspects that should certainly attract more attention for this technique in the near future.

De Prisco et al. used a vibrating technique to encapsulate *L. reuteri* in alginate/chitosan matrixes. The authors report a bead size of 110 ± 5 μm and encapsulation efficiency higher than 90 % for the four systems studied. They also report a 100% of cell survival after freeze drying with no changes on the structure of the beads. The very narrow size distribution reported is an important parameter for homogeneous release of the probiotic in the target site. Additionally, the high survivability of the cells with no changes in the bead structure after freeze drying confirm the efficacy of this drying technique to keep the final material in a powder form [97].

Graff et al. encapsulated *Saccharomyces boulardii* in an alginate/acrylic acid matrix coated with chitosan using the laminar jet break-up. The use of acrylic acid minimized the aggregation and sticking of the beads. In this study the narrow size distribution of the beads was also verified as in the studies of De Prisco et al. confirming the potential of this technique for industrial purposes [98].

Patents and Industry: Extrusion of active ingredients represents only up to 3% when compared to other technologies in the food industry [28].

BRACE GmbH proposes a vibrating nozzle system for the encapsulation of a variety of ingredients. The company holds a patent for production of spherical alginate beads in industrial scale [99]. Based on the BRACE technology, Vésale Pharma developed Intelicaps®, uniform probiotic microcapsules with an alginate-based shell and typical sizes of 600 to 800 μm. The layer-by-layer coating reinforces the pH responsiveness of the system resulting in bead disintegration at pH 5.5 to 6.

Van invented a continuous process for producing controlled release particles containing sensitive components. The particulates are produced at low temperatures and the encapsulation process increases the stability of the active ingredient against moist heat [100], which may be a parameter in the downstream of the industrial processing. Patent owned by General Mills.

Morishita Jintan Co. Ltd produces Bifidobacteria capsules of 2 mm with a three-layer shell of gelatin to be blended with yogurt. The company owns the patent of Asada et al. who created a multilayer capsule composed of cells capable of growing in the confined state. The authors suggest its use in food for treating intestinal disorders or as bioreactors [101].

Doherty et al. produced round droplets of gelled whey protein via the vibrational nozzle technique with further immersion of the beads in an acidic curing solution [102]. The innovative academic work became a start-up, AnaBio Technologies, a company that provides encapsulation expertise for the food, pharmaceutical and animal health care products.

3. Final Valuable Remarks

Encapsulation Interest: Frequently probiotic products, often yogurt, available in the market retain little cell viability at time of consumption. Such viability is strain-dependent and depends on interactions with other species present in the product, culture conditions and production of metabolites affecting the final acidity of the product. In the past, several reports have drawn the attention of scientists to the poor survival of strains in ready-to-consume yogurts [103]. Three out of six samples in a dairy product study contained no traces of live bacteria [28]. These insights highlight the importance of providing protection to bacteria strains before addition to food carriers and consumer consumption.

Cultivation Medium: The most widely used growth medium in academia research to cultivate *Lactobacilli* is the MRS. Despite its effectiveness, the high cost of the ingredients and the non-food-grade label of some of its components turns its use in the industry less of a likelihood. A universal medium called GEM containing just food grade ingredients and composed by glucose, soy peptone, yeast extract, $MgSO_4$ x 7 H_2O and KH_2PO_4 was elaborated by Saarela [66]. The colony forming units (CFU) obtained with GEM were the same or even better than with MRS. For *Lactobacillus rhamnosus*, a supplement of Tween 80 was added, which resulted in better stability during freeze drying and further storage at 37 °C [66].

Industrial secrets and patents: Although many patents are in circulation and can be potentially used by academic researchers to get an industry trend sense, it is of importance to note that there are many unclosed facts within their judicious written language. As a fact, knowledge is transmitted with a patent, but its occurrence deals mostly with legal rights.

Production scale and costs: The production scale and commercialization value of encapsulated ingredients needs to be evaluated when intention of extending academia results into industrialized products is a reality. Continuous or batch production is one of the very first key cost-effective parameters to be taken into consideration. For instance, spray drying can continuously produce microcapsules; on the other hand, emulsion-based techniques are conventionally batch processes. The prices of the technology and raw materials should be also put into question; and a great challenge arises from it. As an example, the customary studied alginate and proteins systems have a low solubility in water and require a costly energetic input to be assessed in the industry [42,46,104]. Product development is also expensive and time-consuming, and generally low margins are achieved with food ingredients in the food industry [46]. Additionally, encapsulation adds another step in the food-processing stream [104]. These are major reasons for higher prices of encapsulated probiotic products. In the end, encapsulated probiotic products are developed for a specific niche market: those who are willing to pay for it.

4. Conclusion

A multitude of research has been done on trying to find suitable polymer matrices along with process technologies. Commonly, natural occurring proteins and polysaccharides are a good polymer choice due to their non-toxicity and biodegradability. The microcapsules must be finely tuned to achieve important goals for the food delivery systems, such as heating and freezing robustness, storage stability and bile and acid-tolerance. Long-term storage imposes a main obstacle for encapsulation of bacteria due to cells subproducts release overtime or lack of nutrients to keep them

alive. Challenges in the fabrication of microcapsules are still manifold and ultimately related to the complexities of bacteria and their response under confinement.

The spray drying, emulsification and extrusion techniques are heavily utilized in the probiotics academic research. The spray drying technique allows the production of small capsules that can be mass-produced, however it damages the cells due to the high temperature applied during the process resulting in poor final cell viability. Emulsification can be scaled up but it can be a costly process that produces capsules varying in size and shape, another drawback. Extrusion, however, utilizes oftentimes mild conditions and the production of narrow size capsules is possible. Electrospraying is gaining more attention for instance, and the vibration nozzle technique is already in the market. Spray coating and spray chilling are continuous processes and overall cost-effective. Both technologies work with mild temperatures translating into fewer bacterial cell injuries. As far as drying techniques, freeze drying under vacuum with cryoprotectants has an interesting potential due to favorable low temperature exposures and superior contamination control. The elevated price of the technique diminishes when cost analysis considers the cell loss in other drying techniques.

Generally speaking, the cost of using encapsulated bacteria in the food industry is still a hurdle and in addition, still a low payload of viable bacteria reaches the gastrointestinal tract. Advances in encapsulation should bridge this gap in the near future. Along these lines, the collaboration between academia and industry could result into successful market novelties.

The research of the gut microbiota is on the radar of different domains. Recent discoveries indicate the impact of the intestine bacteria in respect to the brain functioning and its influence in disorders such as anxiety and depression [105]. Scientists are only starting to understand the diversity, dynamic nature and distribution in this microbiome. Ongoing and future research will contribute to further elucidate the relationship between gut-microbiota and mental disorders. As a result, meaningful novel approaches for their prevention and treatment should emerge one day.

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Conflict of Interest

All the authors declare no conflict of interest in this paper.

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