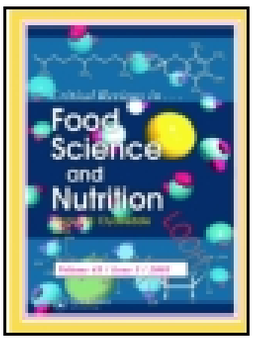

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Physiological Protection of Probiotic Microcapsules by Coatings

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Abstract

Nowadays, food and nutrition have a greater impact in people's concerns, with the awareness that nutrition have a direct impact in health and wellbeing. Probiotics have an important role in this topic and consumers are starting to really understand their potential in health, leading to an increasing interest of the companies to their commercial use in foods. However, there are several

limitations to the use of probiotics in foods and beverages, being one of them their efficiency (directly associated to their survival rate) upon ingestion.

This work is focused in microencapsulation techniques that have been used to increase probiotics efficiency. More specifically, this work reviews the most recent and relevant research about the production and coating techniques of probiotic-loaded microcapsules, providing an insight in the effect of these coatings in probiotics survival during the gastrointestinal phase.

This review shows that coatings with the better performances in probiotics protection, against the harsh conditions of digestion, are chitosan, alginate, poly-L-lysine and whey protein. Chitosan presented an interesting performance in probiotics protection being able to maintain the initial concentration of viable probiotics during a digestive test. The analyses of different works also showed that the utilization of several coatings does not guarantee a better protection in comparison with monocoated microcapsules.

Keywords: Extrusion; Emulsification; Layer-by-layer; Alginate; Chitosan; Poly-l-lysine

1. Probiotics

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit to the host (Food and Agriculture Organization of the United Nations/World Health Organization, 2001). Probiotics have functions that are able to induce a positive effect on human health, such as: a) the production of substances that inhibit pathogen action, blocking pathogenic bacterial cells adhesion sites, b) the promotion of nutrient competition and production, c) the degradation of toxins and toxin receptors and, d) the modulation of immune responses (Prakash *et al.*, 2011). These functions will help in: the reduction of the expression of some biomarkers responsible for colonic cancer; the treatment and prevention of acute diarrhoea in children; the prevention of an initial attack of pouchitis, maintaining remission of ulcerative colitis; the reduction of the symptoms in persons suffering from functional abdominal pain; the improvement of lactose digestion and reduction of symptoms related to lactose intolerance; and the reduction of the risk of necrotizing enterocolitis (Aureli *et al.*, 2011; Sullivan & Nord, 2005).

2. Probiotics market and legislation

There is an increasing demand from consumers for healthy and natural food products, namely functional food products, that are able to provide not only the normal positive impact expected from foods, but also providing consumers with a pleasant, fortified food (Siró *et al.*, 2008; Verbeke, 2005). Functional compounds can be used for the development of a wide range of

functional food products, being beverages and bakery products the leading products where these kind of compounds have been used (Figure 1).

Probiotics are one of the areas of food research and development where more resources are invested. Probiotics' world market was estimated in $ 3.3 billion in 2015; in the last decade more than 500 new probiotic products were introduced in foods' and beverages' markets (Markets and market 2015).

Despite all difficulties for the approval of food claims, a high number of probiotics have been introduced in foods. Some examples are *L. casei shirota* on Yakult and Dannon, *L. reuteri* by Biogaia and *L. acidophilus* by Nestlé (California Dairy Research Fundation, 2015). In Europe, probiotics are approved for use in foods but the European Commission (EC) did not approve yet any claim about their potential health benefits, mostly based on the lack of sufficient scientific evidence (Binnendijk & Rijkers, 2013).

In the United States of America (USA), the Food and Drugs Administration (FDA), divides the utilization of ingredients in four different groups based on their intended utilization and their function in the human body. These four groups are: a) a drug, a new drug or a biological product; b) a dietary supplement; c) a food or food ingredient; and d) a medical food (Degnan, 2008). Despite all differences on approval of probiotics health claims between Europe and USA, their evaluation of probiotics is similar when a claim is directly related to the effect of probiotics in a disease, being mandatory in both countries the approval by their regulatory institutions (EU, 2014; U.S. National Institute of Health, 2012).

However, there are some countries that have already approved health claims on probiotics' benefits. Some of these countries are Japan and Canada. Canada approved general claims considering that microorganisms as *Lactobacillus johnsonii* La 1, *L. johnsonii* Lj 1, *L. johnsonii* NCC 533, *L. rhamnosus* GG and *Saccharomyces boulardii* provide a healthy gut flora and contribute with health benefits to the host. Canada also approved some particular claims that refer the benefits of *L. johnsonii* in the combat of *Helicobacter pylori* infections. *L. rhamnosus* GG and *Saccharomyces boulardii* were also recognized with claims referring the benefits of these organisms in the managing of diseases as infectious diarrhoea, antibiotic-associated diarrhoea and reducing the risk of antibiotic-associated diarrhoea (Canadian Food Inspection Agency, 2015; Hoffmann *et al.*, 2013).

In Switzerland some efforts have been made in the last years regarding the recognition of probiotics health effects. In 2013 two probiotic health claims were accepted for *L. plantarum* LP299V (Vifor SA) and Activia (Danone). In 2014, LC1 (Lactalis) and *Bifidobacterium lactis* HN019 (Dupont) were also the main focus of approved health claims in Switzerland (Switzerland Confederation, 2014). More recently, Yakult has also seen its *Lactobacillus casei shirota* with a health claim approved by Switzerland authorities. These claims are mainly focused on health benefits in the digestion regulation and reduction of residence time (Switzerland Confederation, 2014).

The high investment that has been done in the last years shows that even with the resistance of some regulatory authorities regarding the recognition of probiotics' health effects, they continue to be a food trend with an endless potential to explore. The pressure on food legislators about that subject is increasing as much as scientific favourable opinions of researchers and institutions

are published. However, considering the lack of information about the microbiota and the high difficulty to understand the influence of a probiotic in the human body, the food regulators adopted a more sceptical approach. The need of investment on trials capable to show, without doubts, the effects of these organisms in specific diseases is needed and should be done. While the general recognition of these microorganism as beneficial forms for Human health was already accomplished, the identification of their specific effects should be proved in order to approve health claims.

3. Probiotics encapsulation

According to Gilliland (1989), probiotics should survive during the passage through the upper digestive tract in a large number, to ensure the desired beneficial effects in the host. The minimum suggested concentration of viable cells to provide the benefits mentioned before ranges between 10^8 and 10^9 viable cells per day/dose (Doleyres & Lacroix, 2005; Hou *et al.*, 2003). Probiotics will act after colonization and growth on the intestine and/or colon, being their survival mandatory, until they reach their action spots (Albertini *et al.*, 2010; Chandramouli *et al.*, 2004). Therefore, encapsulation is generally used to improve probiotics survival during digestion, considering the limitations of free probiotics survival (i.e. loss of viability due to the acidic medium).

The main purpose of probiotics encapsulation is their protection against: high oxygen levels (Sunohara *et al.*, 1995); food processing (Tripathi & Giri, 2014); storage; and after consumption (Azizi *et al.*, 2010; Sousa *et al.*, 2013), during the passage through the gastrointestinal system

(Sun & Griffiths, 2000). Other advantages of probiotics encapsulation are the prevention of their interfacial inactivation and the stimulation of production and excretion of secondary metabolites (Nazzaro *et al.*, 2012).

3.1. Encapsulation methods

The incorporation of functional ingredients in foods is complex and in some cases requires, besides the protection of their functionality, the control of their release. Because of that, the encapsulation process chosen will have a very important role in the incorporation of functional ingredients in food products (Cerqueira *et al.*, 2014; Ubbink & Kru, 2006). Microencapsulation (ME) is the "technology of packing solids, liquids and gaseous materials in small capsules that release those contents at controlled rates over long periods of time" (Champagne & Fustier, 2007). This technology can be used to encapsulate probiotics, however during the encapsulation process the viability of the microorganisms should be maintained (Rathore *et al.*, 2013). This means that materials and methodologies used for their production should be carefully evaluated. Other specification is the size of the capsules that some authors mention that must be smaller than 100 µm to avoid a "gritty" sensation when consumed (Hansen *et al.*, 2002; Heidebach *et al.*, 2012). The mouthfeel sensation of capsules in food was evaluated by sensorial analyses and results showed that small (2 – 30 µm), soft and spherical capsules in a lower concentration added to a high viscous gel produce a more pleasant sensation, instead of large (71 – 70 µm), hard, or sharp capsules added in high concentrations to a low viscous medium, which produces a rough and gritty sensation (Engelen *et al.*, 2005; Imai *et al.*, 1995). Moreover, a recent study showed

that capsules with an average size of 30 µm are not detected by sensorial analysis (Heidebach *et al.*, 2012). The solubility of the capsules is an important characteristic because microcapsules should be water-insoluble, to maintain their structure when introduced in foods and beverages, and during the passage through the gastrointestinal system upper part (Ding & Shah, 2007; Picot & Lacroix, 2004) and should be able to release the probiotics in the intestinal track. In fact, microencapsulation technology implies a great knowledge of the characteristics of the materials used (e.g. evaluation of the possible interactions between the functional ingredient with the used material) (Augustin & Sanguansri, 2008), that should be food grade and approved by regulatory authorities. Another issue is the methodology used for probiotics encapsulation (e.g. extrusion, emulsion and spray-drying) that influences probiotics' viability due the conditions used (e.g. temperature). In the next section the most commonly used techniques for probiotics encapsulation will be explained in detail.

Table 1 lists the materials that have been used for encapsulation of probiotics by extrusion, emulsification and spray drying.

The main advantages of encapsulation by extrusion are the simplicity of operation, good performance in a laboratorial environment, lower cost, and high cell viability (Vos *et al.*, 2010). The disadvantages are: the impossibility to produce capsules smaller than 500 µm and greater than 3 mm (Krasaekoopt *et al.*, 2003) by a conventional dropwise method, the process duration and the difficulty of scale-up (Burgain *et al.*, 2011; Liu *et al.*, 2002). To avoid these disadvantages variations to this method were developed, such as the utilization of nozzles instead

of syringe and needle, or the use of emulsions for the production of the microcapsules. When the drop wise method is based in spray systems, such as vibrating nozzles (Chandramouli *et al.*, 2004), air-atomizing nozzles (Cui *et al.*, 2000) and spinning-disk atomization (Senuma *et al.*, 2000) it is possible to produce capsules smaller than 500 µm. More information about the materials used and the sizes and morphologies of the obtained capsules can be found in Rokka and Rantamaki (2010).

Emulsification main advantage is the possibility to create capsules smaller than 100 µm, which is possible due to the small size of the emulsion droplets created, which control the final size (ranged between 25 µm and 2 mm) of the capsules (Heidebach *et al.*, 2009; Mokarram *et al.*, 2009a; Sheu & Marshall, 1993). The main disadvantages are: a) the yield of produced capsules that is very low considering the amount of materials used, during the production (i.e. oil), brings a considerable problem regarding waste, even at a laboratorial scale; b) microcapsules separation from the different phases that may damage the capsules; and c) the high size variation of the produced capsules. Other possible problems are the negative influence of the high stirring rate, the need to form an emulsion, which can influence the probiotics survival, and the impossibility to sterilize vegetable oil if a strict asepsis is mandatory (Gbassi & Vandamme, 2012).

The main advantages of the spray drying method are the relatively low cost of the process, the high production rate of capsules and reproducibility (Burgain *et al.*, 2011; Kailasapathy, 2002). Some of the disadvantages of this process lay on the fact that the technique has a small field of applications due to the high temperatures used during the process. This fact can bring some problems when the aim is the encapsulation of functional compounds, such as probiotics (Burgain *et al.*, 2011). The hot air inlet temperature typically ranges between 150 ºC and 220 ºC,

which decreases during the solvent evaporation process to values between 50 ºC to 80 ºC (Gharsallaoui *et al.*, 2007; Vos *et al.*, 2010). The exposure time of the functional compounds to these high temperatures is of only a few seconds, and generally the core of the microcapsules does not surpass 100 ºC; however these conditions are still a problem for probiotics encapsulation, decreasing their viability (Estevinho *et al.*, 2013; Lian *et al.*, 2002; Rokka & Rantamäki, 2010).

The three encapsulation techniques mentioned above have unique and specific characteristics that suit the encapsulation of probiotics. The extrusion method presents a huge diversity of machines and industrial components able to be adapted to create capsules from different mixtures of polymers and cross linkers. Moreover, these industrial equipment are also able to create capsules' sizes that are not achievable with conventional protocols at a laboratorial scale. The same happens with spray drying, which also presents a great flexibility, although the process temperature is still a huge drawback. At laboratory scale emulsification is clearly one of the most used encapsulation techniques capable of producing capsules smaller than 100 µm, however more studies and investment are needed for the utilization of this technique at large scale.

3.2. Microcapsules coating techniques

Probiotics are usually encapsulated to be used in food, although the current encapsulation techniques (i.e. extrusion, emulsification and spray-drying) are in some cases inefficient regarding the protection of microorganisms, thus decreasing their viability (Rokka & Rantamäki, 2010; Solanki *et al.*, 2013). Alginate is one of the most used materials for microencapsulation,

however alginate-based capsules have porous networks, which allow the exposure of probiotics to the external medium, that is a disadvantage regarding the protection of probiotics in the stomach (Allan-Wojtas *et al.*, 2008). Some works mention that alginate microcapsules, without the application of a coating, have the capacity to protect probiotics during food storage, but not upon the exposure to low pH solutions, such as in the gastrointestinal conditions (Hansen *et al.*, 2002; Sultana *et al.*, 2000). Another problem is the influence of microcapsules' size on probiotic protection, Heidebach and co-workers (2012) refer that only capsules with sizes between 0.2 mm and 3 mm are able to protect probiotics against gastrointestinal harsh conditions. Considering this and that capsules with sizes smaller than 100 μm are preferable regarding sensory aspects, obviously other solutions should be implemented to overcome these limitations.

One of the solutions to improve the performance of microcapsules is the application of a coating on microcapsules surface. This can be done using different materials, which in some cases can be the same material used for capsules production (Krasaekoopt *et al.*, 2003). These coatings will interact with the capsule's surface creating an additional membrane (layer) on the microcapsule (Heidebach *et al.*, 2012) that will improve their performance considering probiotics protection. This coating will decrease capsule's permeability reducing the exposure of probiotics to oxygen, during storage, and improve their stability at low pH and high temperatures (Corona-Hernandez *et al.*, 2013; Heidebach *et al.*, 2012; Mokarram *et al.*, 2009a). In other cases, these coatings were also used to give a new function to the microcapsule, as adhesion properties or to guarantee a controlled release of a micronutrient (Borges & Mano, 2014; Tang *et al.*, 2006).

Considering the protection of probiotics against the harsh conditions of the gastrointestinal system, a huge variety and combination of coating materials have been used. Different

techniques have been used to apply these coatings on probiotics microcapsules, namely layer-by-layer assembly (LbL), performed by the immersion of microcapsules in the polymer solution that will lead to the formation of the coating, and coacervation, where a coacervate is created between microcapsules' surface and a polymer coating.

3.2.1 Layer-by-layer (LbL)

Layer-by-layer technique was first used in the end of the twentieth century to create multilayered films, by the deposition of films in solutions with opposite charges (Decher, 1997; Decher et al., 1992). This technique is based on the layer-by-layer (LbL) principle where two main types of interactions are possible: hydrogen bonding or electrostatic interactions (Fou & Rubner, 1995; Lvov *et al.*, 1995; Sukhishvili & Granick, 2002). LbL is the consecutive adhesion of different materials in a surface, achieved by the consequent assemble of materials with the opposite charge of the surface (Tang *et al.*, 2006). This technique is based on the chemical electrostatic attraction of positively and negatively charged materials (Bertrand *et al.*, 2000). After its use on the production of multilayered films, LbL technique started to be applied on other types of templates with different sizes (e.g. micro- and nanoscale), shapes (e.g. capsules) and chemical compositions (Yan *et al.*, 2014). One of the interesting applications was performed by Champagne and co-workers when they started to applied this technique in microcapsules with loaded probiotics (Champagne *et al.*, 1992; Larisch *et al.*, 1994).

Presently LbL is the more common technique to create a coating on a probiotic microcapsule where the simply immersion of a microcapsule into a biopolymer solution will form a protective coating (Heidebach *et al.*, 2012). Figure 2 shows more specifically this process where a

microcapsule produced by a cationic material (e.g. alginate) is consecutively coated by an anionic material (e.g. poly-l-lysine) and after that by another cationic material. The main forces involved on the utilization of this technique, on probiotics-loaded microcapsules, are electrostatic forces that will form a layer that will coat the microcapsules (Borges & Mano, 2014). This electrostatic interaction is possible through the utilization of negatively or positively charged polymers or biopolymers. Many natural polysaccharides are made of monomers bearing charged groups like amines, sulphates or carboxylic acids (Bertrand *et al.*, 2000), but those compounds must bear a minimal number of charged groups. For this, the pH control, concentration and ionic strength of the polymer solution are fundamental to increase the range between both charged materials that will increase the interaction intensity (Carneiro-Da-Cunha *et al.*, 2011).

An interesting feature of using this technique for the development of a coating is the control of the layer's thickness, which has been studied in some works and shows no relevant increase of capsules size. In microcapsules with approximately 280 µm a thickness ranging between 2 and 3 µm was reported (Tam *et al.*, 2005) and for microcapsules with approximately 40 µm a thickness of 40 nm was obtained (Lin *et al.*, 2008). Cook and co-workers showed that the thickness of a chitosan coating on a alginate microcapsule increases with the immersion time of the microcapsule in the chitosan solution, with a minimal value of 8 µm, after 1 min, and a value of 24 µm, after 2400 min, on capsules with a diameter of app. 1 mm (2011). Other authors concluded that there were no differences between the uncoated and coated microcapsules diameter (Koo *et al.*, 2001). There are several factors that could influence the adhesion of materials during LbL, such as: pH, temperature, ionic strength, adsorption time, polyelectrolyte

molecular weight, polyelectrolyte chain architecture, electrical field, light, mechanical stress, or the addition of other compounds such as proteins or surfactants (Borges & Mano, 2014).

LbL is an easy, efficient and reproducible method of modifying surfaces of different structures (Borges & Mano, 2014). This process is relatively cheap requiring mild conditions and aqueous solutions during the process, working mainly with natural charged materials (Borges & Mano, 2014). Moreover, it is versatile and reproducible, where adhesion times are between 1 and 60 minutes (Bertrand *et al.*, 2000). The main disadvantages of this method are related to the time of adhesion of each layer, that it is not instantaneous (Borges & Mano, 2014). Thus, during the adhesion of consecutive layers a total or partial aggregation of the capsules may occur, which decreases the available surface area for subsequent layer adhesions, decreasing the process efficiency.

3.2.2 Coacervation

Coacervation has been used in microencapsulation of microbial cells (Eratte *et al.*, 2015; Hernández-Rodríguez *et al.*, 2014; Oliveira *et al.*, 2007; Shoji *et al.*, 2013; Zhu *et al.*, 2013), but also in the encapsulation of flavours, preservatives and enzymes (Park & Chang, 2000). A major drawback of coacervation lays in the difficulty in obtaining capsules with small sizes (Freitas *et al.*, 2005; John *et al.*, 2011) and because of that, less emphasis has been given to coacervation techniques on the production of probiotic-loaded microcapsules.

Coacervation is performed by mixing one or more incompatible polymers (simple or complex coacervation) with another incompatible polymer, which will create a phase separation at a specific pH, temperature or composition of the solution. On microcapsules' coatings the

polymers responsible for the connection are the capsule's surface polymer and the solution polymer. To promote the mixture between the two, or more, polymers the dispersion is stirred. After this the parameters mentioned before are changed leading to the separation of incompatible polymer and deposition of the dense coacervate phase surrounding the core material (probiotic microcapsule) (Gouin, 2004). To separate the microcapsules, separation processes such as centrifugation or filtration can be used and the encapsulated material can be also dried by spray or fluidized bed drying (Kailasapathy, 2009). To improve capsule's resistance it is possible to use chemical or enzymatic cross-linking agents (Rathore *et al.*, 2013). This technique has as the most important processing factors the volume of the dispersed phase, the addition rate of the incompatible polymer to the coating polymer solution and the stirring rate of the dispersion (Nihant *et al.*, 1994). Some other factors, such as the composition and viscosity of the coacervate and supernatant phases, can also influence the size distribution, surface morphology and internal porosity of the final microspheres (Nihant *et al.*, 1994).

Some limitations of this technique are related to the complexity of the process, the control of different critical conditions associated with composition and kinetics of reaction, the cost of the process and in some specific cases the evaporation of the core material, dissolution of the core in solvent and its possible oxidation (Madene *et al.*, 2006).

3.3. Materials for the coating of microcapsules

This section provides a description of the main materials used on the coating of probiotic-loaded microcapsules and their performance (Table 2). The main results regarding the comparison of the

uncoated and coated probiotic microcapsules in gastrointestinal simulation media will only be discussed in order to analyse if there is a positive or negative effect of the coating on the probiotic survival in those media. Other works besides those presented in Table 2 were also analysed (see e.g. Fareez *et al.*, 2015; García-Ceja *et al.*, 2015; Lee *et al.*, 2004; Li *et al.*, 2011; Martoni *et al.*, 2007; Mi *et al.*, 2013a; Mokarram *et al.*, 2009b; Sohail *et al.*, 2011; Zou *et al.*, 2011).

3.3.1. Alginate

Alginate is a polysaccharide derived from brown algae or bacterial sources constituted by 1→4 linked β-(D)-glucuronic (G) and α-(L)-mannuronic (M) acids (Rinaudo, 2008). The main reasons for its high utilization on probiotics microencapsulation are its GRAS (generally regarded as safe) status being used as a food additive worldwide, its lack of toxicity (Gombotz & Wee, 1998), its strong capacity to be cross-linked and the different mild gelling characteristic which change with the molecular weight and ratio between M and G acids (Thu *et al.*, 1996). Alginate, due to the presence of carboxylic groups on both monomers, has a negative charge above its pKa (3.3 - 3.5) (Cook *et al.*, 2012), as presented in Figure 3.

Furthermore, one of the characteristics that is interesting regarding the creation of microcapsules is the high affinity of alginate residues to divalent metals, such as calcium, cadmium, zinc and barium (Draget *et al.*, 2000). With these interactions an "egg-box" structure is formed, where happens the consecutive connection of four alginate G residues to one metal ion, which brings more stability and swelling capacity to the microcapsule, being the affinity of these metals bigger with G residues than with M residues (Thu *et al.*, 1996). However, cations such as sodium and

magnesium have an anti-gelling influence in alginate as they replace calcium in the matrix (Lee *et al.*, 2004). Equally, chelating agents such as phosphate can also contribute to the physical instability of the microcapsules by competing with the matrix for the capture of calcium ions (Krasaekoopt *et al.*, 2006a). Other disadvantage of using alginate is its sensitiveness to acidic media, being a concern when used to protect bacteria against harsh stomach conditions (Burgain *et al.*, 2011). In some cases alginate is mixed with other polymers, such as starch, to improve their resistance against acidic media and thus increase their probiotic protection capacity (Hansen *et al.*, 2002; Krasaekoopt *et al.*, 2003; Sultana *et al.*, 2000; Sun & Griffiths, 2000).

Alginate is not the most used material to coat probiotic-loaded microcapsules, even though some works have presented alginate with this functionality. Annan and co-workers presented an increased survival of 1.64 log CFU after the exposure of *Bifidobacterium adolescentis* 15703T to gastric and intestinal simulation media when experiments were performed with an alginate coating, in comparison with an uncoated alginate microcapsule (Anan *et al.*, 2008). In other work, Krasaekoopt *et al.* (2004) used the alginate coating on an alginate microcapsule and showed an increase of the bacteria survival on 5, 3 and 3 log CFU (*Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994, and *Lactobacillus casei* 01, respectively) compared with the uncoated microcapsules. However, other authors (Brinques & Ayub, 2011; Iyer & Kailasapathy, 2005) also showed that the utilization of alginate as a coating did not have a relevant positive impact on bacteria's survival.

Comparing the results above, it is clear that the effect of alginate can be positive or not relevant, as a coating in different works, considering probiotics protection. However, it is important to understand in which situations an alginate coating can have a positive impact. Analysing the data

on Table 2, about the works mentioned in the last paragraph, the most relevant work that shows a considerable benefit about alginate's utilization is Krasaekoopt *et al.* (2004). To explain these differences it is important to analyse each variable by itself, to understand where are differences that might explain these results, in different works using the same coating. Brinques & Ayub (2011) and Iyer & Kailasapathy (2005) used the same capsule producing technique (extrusion), the same coating method (LBL), the same concentration of alginate in capsules' core and in the coating, and even on Brinques and Ayub's (2011) work the same probiotic was used (*Lactobacillus acidophilus*). Because of that, the differences in those four works might be justified by the differences in capsules' sizes and in the media used during *in vitro* tests. The capsules used by Krasaekoopt *et al.* (2004) were significantly larger than the ones used in the other works, which provided a higher surface for coating adhesion and therefore a higher mass of alginate coating the capsule. This higher amount of alginate could have had a positive impact on probiotics protection. Other variable that can justify these differences is the type of medium used to simulate the stomach conditions by Krasaekoopt *et al.* (2004), who used a smaller ionic strength compared with the other mentioned works.

3.3.2 Chitosan

Chitin is a natural, linear cationic polysaccharide with glucosamine and *N*-acetyl glucosamine residues presented in the shells of crustaceans, molluscs, the cell walls of fungi and the cuticle of insects (Kumar, 2000). Chitosan has a heterogeneous distribution of acetyl groups along the chains related with their origin, chitin, that has a semi crystalline morphology (Rinaudo, 2006). In solution, chitosan behaves as a cationic polyelectrolyte (Peniche *et al.*, 2003) with amine residues presents at a pKa around 6.5 and a positive charge in pH's below than that (Sogias *et*

al., 2010). Chitosan is insoluble at pH higher than 5.4, which can be influenced by the acetylation degree (Huguet *et al.*, 1996). In Figure 4 is presented its structure as its functional groups.

Chitosan obtained from animals is not approved in EU as food additive but presents the GRAS (Generally Recognized as Safe) status in USA. However, if obtained from fungi (*Aspergillus Niger*), it is approved for wine processing aid in the European Union (EU, 2012), is GRAS under US FDA regulation (FDA, 2011) and is approved as food additive in Japan (JFCRF, 2011). Moreover, chitosan obtained from fungi is approved as additive on the production of wine, beer, cider, spirits and food grade ethanol by Food Standards by the Australian and New Zealand legislation (FSANZ, 2013).

One of the problems of using it as the core material in encapsulation is its inhibitory effect against some bacteria (Groboillot *et al.*, 1993). Nevertheless, due to its cationic behaviour and capacity to resist to acidic media, chitosan is one of the most used materials as a coating, when considering the utilization of coatings to protect probiotics against the harsh gastrointestinal conditions.

Cook and co-workers (Cook *et al.*, 2011) evaluated the influence of a chitosan coating on an probiotic-loaded alginate microcapsule when exposed to an acidic medium. Results showed that *Bifidobacterium breve* NCIMB 8807 survived in an alginate microcapsule with a final count of 5.2 ± 0.8 log CFU (from an initial count of 9.5 log CFU), while with the utilization of a chitosan coating on alginate microcapsules a final count of 7.3 ± 0.2 log CFU was obtained in the same acidic conditions, showing an improvement of 2.1 ± 1.0 log CFU on bacterial survival. Authors

concluded that chitosan could work as a buffer, reducing the effect of the acid on bacterial viability, through the reduction of the microcapsules' permeability to the acidic medium, while also maintaining their integrity and thus decreasing the release of bacteria. Also De Prisco and co-authors (2015) observed that the utilization of a chitosan coating improved the survival of *Lactobacillus reuteri* DSM 17938 on gastric and intestinal media. They showed that there was no significant reduction of the viability of encapsulated bacteria in coated microcapsules after being subjected to those media, while for capsules without any coating a reduction of 0.35 log CFU on viable bacteria was observed when subjected to the acidic medium. Similar findings were presented by Iyer and Kalasapathy (2005) when they used a chitosan coating on alginate microcapsules to protect *Lactobacillus acidophilus*. Starting from an initial count of 9.2 log CFU, they obtained a final count of 9.1 log CFU when using chitosan coated microcapsules and a final count of only 6.3 log CFU for the uncoated microcapsules (thus a very significant 2.8 log CFU difference between the two). The same results were achieved by other works using alginate microcapsules coated with chitosan (Darjani *et al.*, 2016; Fareez *et al.*, 2015; García-Ceja *et al.*, 2015; Wunwisa Krasaekoopt *et al.*, 2004; Lee *et al.*, 2004; Mi *et al.*, 2013b; Sohail *et al.*, 2011; Zou *et al.*, 2011).

Other authors also worked with chitosan but no positive influence was achieved by the presence of a chitosan coating, considering *Lactobacillus plantarum* BL011 protection (Brinques & Ayub, 2011). Graff and his co-workers (Graff *et al.*, 2008) used chitosan coating for the protection of *Saccharomyces boulardii*, although in this work there was no evidence of an improvement of the protective properties of the alginate microcapsule with that material. Krasaekkopt and Watcharapoka (2014) used chitosan as a coating for alginate microcapsules and obtained a low

performance considering the protection of *Lactobacillus acidophilus* and *Lactobacillus casei*. The initial and final viable count have a 6 log CFU gap which considering the size of the capsules and the medium used for the gastric simulation, showed a low performance when compared with other works.

From the presented works it is clear that the use of a chitosan coating is one of the means to protect probiotics from gastric conditions and to achieve a controlled delivery in the intestine. It is also important to mention that some studies do not show this capacity, although there are no explanations for these differences. However it is known that biopolymers' characteristics (i.e. molecular weight, deacetylation degree) and other experimental conditions have a great influence on the behaviour of coated microcapsules, and should be considered.

3.3.3 Poly-l-lysine (PLL)

Poly-l-lysine is a cationic natural, non-ribosomal homo-poly(amino acid), so this non-peptide is constituted solely by one type of amino acid in its backbone. This form is naturally produced by *Streptomyces* bacteria (Takehara *et al.*, 2008). This poly(amino acid) is constituted by 25-35 L-lysine residues (Hamano *et al.*, 2013). Its isoelectric point is approximately 9, being thus a positively charged material below this pH, mainly because of its cationic groups such as NH_3^+, as presented in Figure 5 (Orive *et al.*, 2006; Yoshida & Nagasawa, 2003).

PLL is commercially produced worldwide by a modified *S. albulus* and used as food preservative due to its antimicrobial activity against a large spectrum of bacteria and fungi (Hamano *et al.*, 2013); due to its food-grade status it is used as a food additive in South Korea (Korea & Province, 2014), USA (FDA, 2003) and Japan (Yoshida & Nagasawa, 2003). This

material has also been used as a layer on delivery systems applied to medical and pharmacological purposes (Mekhail *et al.*, 2014; Santos *et al.*, 2012), as well as in probiotic protection.

PLL's active properties and charged behaviour has led to the interest to test it as a coating of probiotic-loaded microcapsules. However, its positive behaviour on probiotic protection is not clear. Cui and co-workers (2000) used a PLL coating on alginate microcapsules for *Bifidobacterium bifidus* protection and compared their survival in a gastric fluid simulation medium, showing no statistically significant differences between the probiotic survival in coated and uncoated microcapsules. Also Zou *et al.* (2011) presented a comparison between an uncoated alginate microcapsule and a PLL-coated alginate microcapsule and did not observe significant differences between the two microcapsules regarding the protective behaviour towards *Bifidobacterium bifidum F-35* in simulated gastrointestinal tract media. On the other hand, Iyer and Kalasapathy (2005) showed that the utilization of PLL as a coating on alginate microcapsules did have a small relevant positive impact on bacteria survival, once the final counts for surviving bacteria were of 7.3 and 6.3 log CFU for the experiments with PLL coating and without coating, respectively. In another work a similar behaviour was observed, and the utilization of PLL and a palm oil mixture as a coating of alginate microcapsules showed a positive impact on bacteria survival decreasing by 1 log the bacterial death (*B. lactis type Bl-O4 and B. lactis type Bi-07*); however there were other tested bacteria for which no statistically significant differences were observed between coated and uncoated alginate microcapsules (Ding & Shah, 2009b).

The apparent contradiction between results reported by different authors is rather common in this area. The fact is that often the works differ in some details which are important. As an example, Cui and co-workers (2000) used pepsin during the stomach phase while Iyer and Kalasapathy (2005) did not. This is sufficient to justify why the first group of authors found no statistically significant differences while the second group of authors reported an improvement of 1 log on the survival of probiotics: it is known that pepsin affects the PLL coating, thus reducing its capacity to protect probiotics against the harsh conditions of the gastric medium. In any case, even when intact, PLL coatings show a rather high porosity which facilitates the entrance of acidic media and significantly reduces probiotics' survival in the stomach, thus explaining the modest improvements of probiotics survival reported by the majority of the works.

3.3.4 Whey protein

Milk proteins (e.g. whey proteins and casein) can be used to encapsulate probiotics producing a high-density gel network that can protect probiotic strains. Considering the extreme conditions of stomach, these proteins are able to create a higher local pH-value within the protein matrix of the capsules, caused by the proteins' buffering capacity (Vidhyalakshmi *et al.*, 2009). Milk proteins have excellent gelation properties and they also are biocompatible with probiotics (Livney, 2010). More specifically, whey proteins are often used because of their amphoteric character, being commonly mixed with negatively charged polysaccharides such as alginate, carrageenan or pectin. Above their isoelectric point, these structures change their net charges to positive, causing an interaction with negatively charged polysaccharides (Guérin *et al.*, 2003). Whey proteins usually have their isoelectric point at pH 5.2 (Ju & Kilara, 1998), at which aggregation occurs.

Through a coacervation method Gbassi and co-workers (2009) developed a whey protein coating for three different types of *Lactobacillus plantarum* encapsulated in alginate microcapsules. Positive results were accomplished with the utilization of this material as a coating, where without the coating there was a complete inactivation of the bacteria after 90 min. With whey protein there was a considerable survival at 120 min, in the range of 5-7 log CFU/g, between the three different bacteria/experiments. After 180 min of exposure in the simulated intestinal medium a survival ranging between 3-4 log CFU/g was achieved, showing that whey proteins may be used to enhance bacterial survival in harsh conditions.

However, the use of this kind of coatings is not consensual, and can be influenced e.g. by the microcapsule material. Gebara and co-workers (2013) performed an experiment with the utilization of whey protein as a coating for pectin microcapsules. The results comparing the bacteria survival encapsulated in microcapsules with and without the whey protein coating did not show statistically significant differences. These tests are difficult to compare considering that several variables might influence the results. Besides the variables listed in each column of Table 2, there are other factors that may contribute to reduce probiotics' viability; these include the physiologic state of probiotics when encapsulated, the methods used to isolate the capsules after their production and the methods used to destroy the capsules for viability analyses.. This means that a variety of results using exactly the same procedures and the same probiotic can be expected.

3.3.6 Final remarks on coating materials

Considering alginate as a coating, it is important to mention that it is not the best material to coat probiotic-loaded microcapsules regarding probiotic protection. This is justified by its gelling properties and its high porosity, that facilitate permeability (i.e. penetration of the acidic medium) and with this the contact of probiotics with harsh external conditions. Nevertheless, alginate should be used where its properties provide more relevant advantages, that is, as a microencapsulation material. As well as alginate, PLL was used in some works and showed that it does not have a strong capacity to be used as a microcapsule coating for probiotics protection against harsh media. Due to its strong capacity to interact with alginate microcapsules, because of its positively charged state above pH 9, PLL utilization brings a potential functionality for LbL assembly. It would thus be interesting to perform further studies in order to understand PLL's capacity to provide other functions to the coated microcapsules.

Chitosan showed to be the most interesting material to protect microencapsulated probiotics, with good results in different alginate microcapsules (produced by different techniques and with different types of alginate), probiotics strains and environmental conditions. It is also important to notice that in all presented works the core was mainly constituted by alginate.

Considering the different techniques used to coat microcapsules it is interesting to notice that alginate is used in most cases as a second microcapsule and not as a coating. On the contrary, chitosan and PLL, both positively charged compounds, are almost always used as a coating by LbL assembly, while whey proteins are in all cases used as a coating by complex coacervation. Comparing the two main coating methodologies it is possible to conclude that the more

important factor is the type of material used (excellent results on probiotic protection by chitosan and not so positive with PLL).

Table 2 clearly shows that it is extremely difficult to compare results from different works, mainly when so many variables might influence them. However, within the same work, comparisons are possible and were presented in Table 2 (Brinques & Ayub, 2011; Iyer & Kailasapathy, 2005; Krasaekoopt *et al.*, 2004; Martoni *et al.*, 2007; Zou *et al.*, 2011). It is important to mention that when the performance of a given material is reported as being good or bad, it does not mean that it would be the same under different conditions/applications. Thus the main conclusion here is that each material may have potential and it is important to analyse the combination of materials, techniques and tests performed in a case-by-case basis.

3.4. Microcapsules with two or more coatings

Some works also report the utilization of more than one coating, in order to improve probiotics survival during the passage by the gastrointestinal harsh conditions. The adhesion of a second coating is only possible by subsequent microencapsulation or through the utilization of the LbL assembly technique.

The protection capacity of PLL and alginate (first and second coating, respectively) was compared with a unique alginate coating (Krasaekoopt *et al.*, 2004) in alginate microcapsules. On the three bacteria studied (*Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994, *and L. casei* 01) the system that always presented better protection of the bacteria was the one with only one coating of alginate (with an increased survival between 1-3 log CFU, depending on the bacteria tested). However, the alginate/PLL/alginate (APA) system showed a

better performance than the uncoated microcapsules. Results showed that the utilization of the APA system for *L. acidophilus* and *L. casei* allowed an increase of survival of 4 log CFU and 1 log CFU, respectively, and for *B. bifidum* no significant differences were observed. In another work where an APA system was used to protect *L. plantarum*, results showed an increasing survival of 6 log CFU (with a starting count of 10 log CFU) when compared with the uncoated *L. plantarum*-loaded microcapsules. However, there are no results considering the uncoated system, or even the system with just a single coating, thus making it impossible to compare these results with previous works (Martoni *et al.*, 2007).

The results discussed before were similar on, the single and double coating showed an increase of the protective effect compared with the uncoated microcapsule on the gastric simulation (1-3 log increase on survival), although on the intestinal tests just the double coating made a difference (1-2 log increase on survival). Comparing the results of microcapsules coated with one and two coatings it is possible to understand that the microcapsules with two coatings presented a better performance on the acidic environment than the monocoated microcapsules.

In conclusion there is no evidence that a higher number of coatings could perform a better protection when compared with microcapsules with a single coating, but more works with more than a single coating are needed to clarify this issue.

4. Conclusions and future perspectives in probiotic encapsulation

Without any doubt one of the research trends in this area is to find industrial encapsulation technologies that guarantee the survival of probiotics.

When considering the issues mentioned before about cells survival in spray drying encapsulation technique, due to overheating, spray chilling appears as one of the alternatives for probiotic encapsulation. Spray chilling has the same approach as spray drying but using a cold conveying air cold chamber, instead of hot air (Champagne & Fustier, 2007; Pedroso *et al.*, 2012). Electrodynamic processes, such as electrospraying and electrospinning, can be also used for the encapsulation of probiotics. The main advantages of these techniques are their capacity to create very thin fibres or capsules that could be obtained in different scales. These techniques start to be used in the food area, namely on packaging materials and encapsulation of bioactive compounds, and more recently also for probiotics microencapsulation (Laelorspoen *et al.*, 2014; López-Rubio *et al.*, 2012).

Considering the materials used in microencapsulation, a major concern has been the utilization of food grade ingredients and in some cases food itself as the main material for encapsulation. Some examples are the utilization of goat's milk (Ranadheera *et al.*, 2015), pea protein (Kent & Doherty, 2014), peanut butter (Klu & Chen, 2015) and chocolate (Champagne *et al.*, 2015; Kemsawasd *et al.*, 2016). The utilization of food ingredients as the main material of a microcapsule could be an interesting way of creating a more natural core shell, from a consumer's point of view, and a more homogeneous food product. Something similar is happening but with the utilization of prebiotics as the main microcapsule material instead of encapsulating them together with probiotics. Some examples are the use of fructooligosacharides (Rajam & Anandharamakrishnan, 2015), native rice starch and inulin (Avila-Reyes *et al.*, 2014). The utilization of new materials can also improve the performance of conventional systems,

being the utilization of new sources of natural ingredients very important to the development of this area.

Innovation on probiotics microencapsulation has been achieved not only through the development of new industrial/laboratorial equipment but also by the utilization of methodologies/technologies used in other fields of knowledge. There are also other important perspectives in the utilization of probiotic microcapsules, e.g. in some cases microcapsules are used as a continuous microreactor able to be a continuous producer of micronutrients in the human gut (Ramos, *et al.*, 2016; Ramos, *et al.*, 2016).

The actual trend is to focus on precision and customization in food production. Thus, food processing in general, and specifically microencapsulation, is evolving to the utilisation of state-of-the art technologies such electrospinning or even 3D printing, pointing at the need to maintain research efforts in this attractive and promising area of knowledge.

However, more research efforts are needed in order to address the capacity of some materials for probiotics protection. More than that, a standardization of the gastrointestinal simulation procedures would be very important to compare and conclude which materials and techniques could suit better the needs of each probiotic and food system.

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Table 1 - Polymers used for probiotics encapsulation by extrusion, emulsification and spray drying.

Material	**References**
Extrusion	
Sodium alginate	(Etchepare *et al.*, 2016; Smidsrd & Skjak-Brae, 1990)
k-carrageenan	(Büyükgüngör, 1992; Tsen *et al.*, 2008)
Xanthan gum	(Jiménez-Pranteda *et al.*, 2012; McMaster & Kokott, 2005)
Gellan	(Jiménez-Pranteda *et al.*, 2012)
Whey protein	(Doherty *et al.*, 2011; Guérin *et al.*, 2003)
Emulsification	
Sodium alginate	(Holkem *et al.*, 2016; Sheu & Marshall, 1993)
k-carrageenan	(Adhikari *et al.*, 2003, 2000)
Chitosan	(Groboillot *et al.*, 1993; Peniche *et al.*, 2003)
Gelatine	(Hyndman *et al.*, 1993)
Caseinate	(Crittenden *et al.*, 2006; Würth *et al.*, 2015)
Sesame oil	(Hou *et al.*, 2003)
Spray drying	
Sodium alginate and Carrageenan	(Burey *et al.*, 2009)
Starch, Arabic gum, Gelatine, Whey protein, Pea protein and Maltodextrin	(Arslan *et al.*, 2015; Pinto *et al.*, 2015)

Skim milk	(Gardiner *et al.*, 2002; Maciel *et al.*, 2014)
Fructooligosacharide	(Rajam & Anandharamakrishnan, 2015)
Cellulose acetate phthalate	(Antunes *et al.*, 2013)

Table 2 - Coated probiotic microcapsules subjected to an *in vitro* gastrointestinal simulation

Microencapsulation and coating technique	**Microcapsule material (CL: cross-linker)**	**Coating material**	**Bacteria**	**Capsule size (µm)**	**Stomach conditions**	**Intestinal conditions**	**Initial count (IC) and experiment without coating (WC)**	**Final amount in coated microcapsules**	**References**
Extrusion; LBL assembly (*)	Alginate (2%) CL: 0.05 M $CaCl_2$	Chitosan (0.4%)	*B. breve* NCIMB 8807	n.a.	Simulated gastric juice (pH=2), 120 min	PBS, pH 7.2 (180 min)	(log CFU/mL) IC - 9.5 WC (G; I) - 2.0; 2.5	(log CFU/mL) (G; I) 7.3; 6.8	(Cook *et al.*, 2011)
Extrusion; LBL assembly (*)	Alginate (2%) CL: 0.5 M $CaCl_2$	Chitosan (0.7%)	*L. reuteri* DSM 17938	110 ± 5	0.3 % Pepsin 0.5% NaCl (pH=2.5), 180 min	5 g/L of bile solution (240 min)	(log CFU/mL) IC - 9.40; WC (G; I) - 9.15; 9.3	No relevant differences	(De Prisco *et al.*, 2015)
Emulsion; Extrusion or emulsion (*)	Gelatin (13%) CL: genepin 1.25 mM	Alginate (1%) (previous adhesion of Ca^{2+} ions)	*B. adolescentis* 15703T	49.0-53.1	Pepsin on 0.2% NaCl solution (pH=2), 60 min	Pancreatin (1 g/L), bile salts (4.5 g/L), pH 7.4, 240 min	(log CFU/mL) IC - 9.60; WC (G; I) 7.13; 6.71	(log CFU/mL) (G; I) 8.92; 7.35	(Annan *et al.*, 2008)
Emulsion; LBL assembly (*)	Alginate (3%) CL: 0.05 M $CaCl_2$	A - Alginate (0.17%) B - Chitosan (0.4%)	*L. plantarum* BL011	n.a.	Pepsin on NaCl 0.5% solution (pH=2), 120 min	pancreatin (1g/L), NaCl (0.5%, w/v), 4.5% bile salts, pH 8.0, 120 min	(log CFU/mL) IC - 9 (app) WC (G; I) - A and B - 5.2; n.a.	(log CFU/mL) (G) A - 4.8 B - 5.5	(Brinques & Ayub, 2011)
Spray dryer; LBL assembly (*)	Alginate (1.5%) CL: 0.2 M $CaCl_2$	Poly-l-lysine (0.02%)	*B. bifidus*	80-130	pH 1.5 without pepsin, 120 min	Intestinal fluid (pH 6.8), 12h	(log CFU/g) IC - 9.7; WC (G; I) - 7.7; n.a.	(log CFU/g) (G) 7.4	(Cui *et al.*, 2000)
Emulsion; LBL assembly (*)	Alginate (3%) CL: 0.01 M $CaCl_2$	1st coating: Palm oil (pure) 2nd coat.: Poly-l-lysine (0.05%)	*B. lactis* type Bl-O4 (A), *and B. lactis* type Bi-07 (B)	35-38	pH 2.0 with 5.0 M HCL, 120 min	n.a.	(log CFU/mL) Log 9 (app) WC (G): 4.27 (A); 5.93 (B).	(log CFU/mL) (G) 6.27 (A); 6.69 (B).	(Ding & Shah, 2009a)
Extrusion; Complex Coacervation (*)	Alginate (2%) CL: 0.1 M $CaCl_2$	Whey protein (2%)	*A - L. plantarum* 299v, B - L. plantarum 800 and C - L. *plantarum* CIP A159	n.a.	9 g/L NaCl and 3 g/L Pepsin (pH 1.8), 120 min	9 g/L NaCl, pancreatin and trypsin (10 g/L) 3 g/L of bile salts (pH 6.5), 180 min	(log CFU/g) IC: A - 10.04; B - 10.00; C - 10.12; WC: No survival after 90 min (G)	(log CFU/g) (G; I) A – 7; 3 B - 5, 3 C - 5.4; 4	(Gbassi *et al.*, 2009)
Emulsion; Complex coacervation (*)	Pectin (2%) Butter (2%) CL: 2% $CaCl_2$	Whey protein (4%)	*L. acidophilus La5*	n.a.	KCl (1.12 g/L); Mucin (3.5 g/L) pepsin (0.26 g/L)	Acidic medium + pancreatin (1.95 g/L) (pH 7.0), 300	(log CFU/mL) IC - 6.96; WC: (G; I) 6.28; 5.45	(log CFU/mL) (G; I) 6.05; 5.22	(Gebara *et al.*, 2013)

					(pH 3.0), 120 min	min			
Extrusion; LBL assembly (*)	Alginate (2.5%) CL: 0.1 M $CaCl_2$	Chitosan (0.4%)	*Saccharomyces boulardii*	356±10	0.1 M HCl (pH 1.1), 120 min	pH 6.8 phosphate buffer for 2 h	(log CFU/g): IC - 8 WC (G; I): 8; 10	(log CFU/g) (G; I) 8; 10	(Graff *et al.*, 2008)
Extrusion; LBL assembly (*)	Alginate (1.8%) CL: 0.1 M $CaCl_2$	Chitosan PLL Alginate	*L. acidophilus* CSCC 2400 or CSCC 2409	500	0.5 M HCl (pH 2), 3h	Milk-yeast extract medium (pH 6.9) 1.0% bile salts 6h)	(log CFU, app) IC - 9.2 WC: 6.3	(log CFU, app) Chi: 9.1 PLL: 7.3 Alg: 6	(Iyer & Kailasapathy, 2005)
Extrusion; LBL assembly (*)	Alginate (2%) CL: 0.5 M $CaCl_2$	Chitosan (0.4%)	A - *L. acidophilus 5 and* B - *L. casei 01*	1830-1850	0.08 M HCl containing 0.2% NaCl, pH 1.55, 120 min	0.05 M KH_2PO_4, pH 7.43; 0.6% bile salt, 150 min	(log CFU/mL) IC: A - 9.4; B - 8.7 WC: n.a.	(log CFU/mL) (G) A - 2.7 B - 2.3	(Krasaekoopt & Watcharapoka, 2014)
Extrusion; LBL assembly (*) (**)	Alginate (2%) CL: 0.5 M $CaCl_2$	Alginate (0.17%)	*L. acidophilus 547 (A), B. bifidum* ATCC 1994 (B), *and L. casei* 01 (C)	1890	0.08 M HCl containing 0.2% NaCl, pH 1.55, 120 min	0.05m KH_2PO_4, pH 7.43, 0.6% bile salt solution, 2h	(log CFU/mL) IC - 9; WC (G; I): A, B and C - 3; A – 5; B – n.a.; C – 3	(log CFU/mL) (G; I) A - 8; 4 B – 6; n.a. C- 6; 3	(Krasaekoopt *et al.*, 2004)

G - gastric simulation experiment; I - Intestinal simulation experiment; (*) – mono-coating experiments; (**) - two or more coating coatings

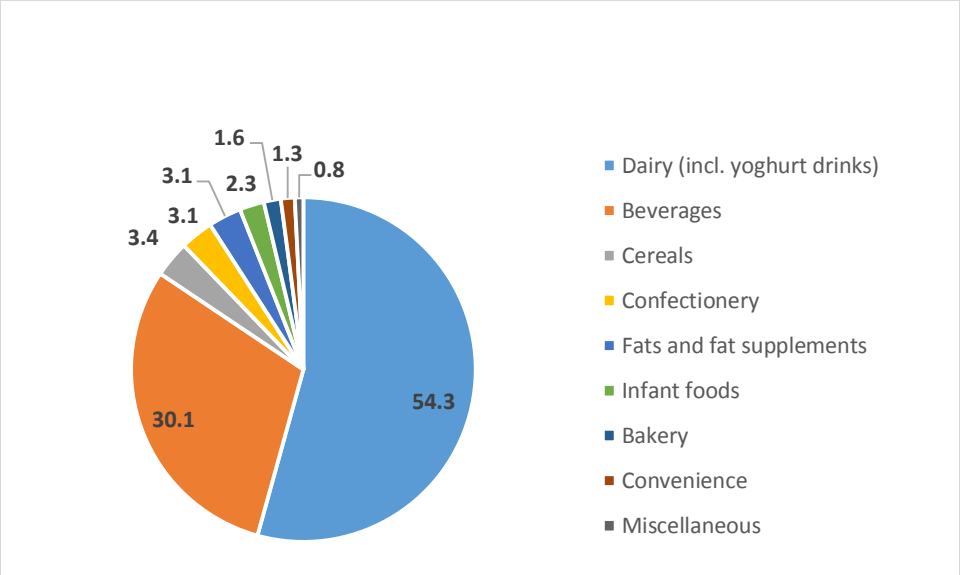

Figure 1 - Functional food products on the European market in 2008 by sectors. Adapted from Stein & Rodríguez-Cerezo (2008).

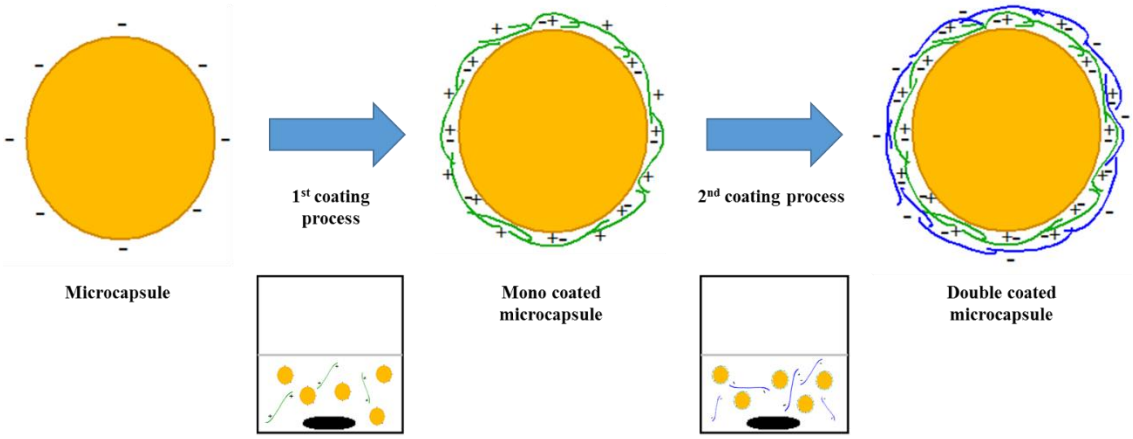

Figure 2 - Layer-by-layer technique scheme on probiotic microcapsules - a two-layers' construction, where the original microcapsule is constituted by an anionic material.

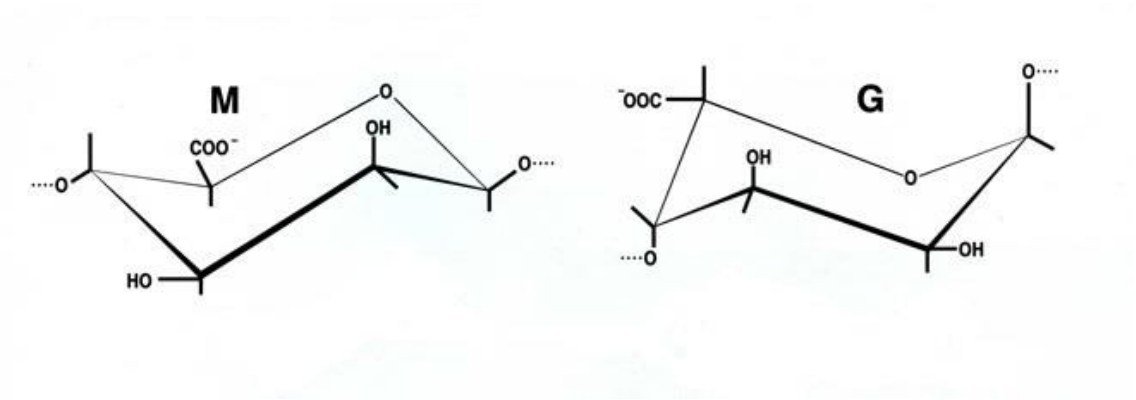

Figure 3 – Representation of mannuronic (M) and glucuronic (G) acids that are responsible for alginate's structure.

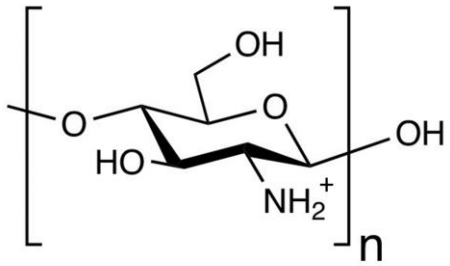

Figure 4 - Chitosan structure - positive charges of the amine group are presented.

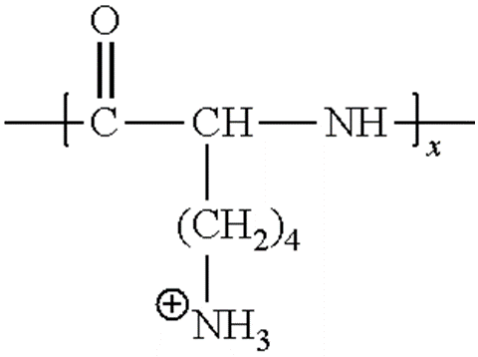

Figure 5 - Poly-l-lysine chemical structure and its charged amine group.