Encapsulation of *Lactobacillus casei* and its stability in model foods

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Thesis to obtain the Master of Science Degree in Biological Engineering

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Neste projecto optimizou-se o método de encapsulação de probióticos usando o método descrito por Thomas Heidebach e a sua equipa. Este método usa as propriedades de gelificação das proteínas do leite, nomeadamente da caseína, e uma emulsão de água em óleo para produzir microcápsulas contendo *Lactobacillus casei* LAFTI L26. Fizeram-se vários ensaios com diferentes emulsificantes (sem emulsificante, 4% p/p de lecitina, 0,5% p/p de lecitina e 0,5% p/p de *emulgator D*) e com diferentes condições de agitação (500 rpm e 1000 rpm) para determinar qual a influência destes parâmetros no diâmetro médio das cápsulas produzidas. Após estes ensaios determinou-se que a melhor formulação para a produção de cápsulas era o uso de 0,5% p/p de lecitina, obtendo-se cápsulas com um diâmetro médio de 52 µm e com forma esférica. Terminada a etapa de optimização do método de encapsulamento, estudou-se a estabilidade das cápsulas em condições simuladas do tracto gastrointestinal e armazenadas em leite, iogurte e queijo. Ao estudar a estabilidade das cápsulas produzidas em condições simuladas do tracto gastrointestinal foi possível concluir que, quer as cápsulas produzidas com 0,5% p/p de lecitina, quer as cápsulas produzidas com 0,5% p/p de *emulgator D* são mais estáveis que as células livres de *L. casei*. No início deste ensaio o número de células viáveis de *L. casei* para as cápsulas com lecitina e *emulgator D* e para as células livres era entre 8 e 9 log CFU mL\(^{-1}\). Ao fim de 6 horas, o número de células viáveis era cerca de 5 log CFU mL\(^{-1}\) para as cápsulas com lecitina e *emulgator D*, e aproximadamente 0 log CFU mL\(^{-1}\) para as células livres. No estudo da estabilidade das cápsulas em leite, iogurte e queijo, tanto as cápsulas como as células livres mantiveram-se estáveis ao longo das 4 a 5 semanas de armazenamento, sendo possível concluir que as cápsulas não são fundamentais na protecção das células livres. Realizaram-se ainda estudos preliminares para avaliar se o *scale-up* do processo de encapsulamento seria viável. Apesar de aparentar ser possível o *scale-up* do processo, os estudos realizados não foram suficientes e por isso são inconclusivos.

**Palavras-chave:** *Lactobacillus casei* LAFTI L26; encapsulamento por emulsão; estabilidade das cápsulas; tracto gastrointestinal.
Abstract

In this project, the encapsulation method by means of rennet-gelation of milk proteins described by Thomas Heidebach and co-workers was optimized to produce capsules containing *Lactobacillus casei* LAFTI L26. This method uses the gelation properties of milk proteins, especially casein, and a water in oil emulsion to form microcapsules. Several experiments were performed with different emulsifier concentrations (no emulsifier, 4% w/w of lecithin, 0.5% w/w of lecithin and 0.5% w/w of emulgator D) and different agitation speeds (500 rpm and 1000 rpm) to evaluate their influence on capsules’ average diameter and partial size distribution. After these experiments it was possible to conclude that the best capsules produced were obtained using 0.5% w/w of lecithin at 500 rpm, had around 52 µm of volume based median diameter and spherical shape.

The capsules stability was studied in simulated conditions of the gastrointestinal tract and stored in different dairy products during long-term storage. In simulated conditions of the gastrointestinal tract, the initial viable cell numbers were set between 8 and 9 log CFU mL$^{-1}$ for capsules with 0.5% w/w of lecithin, 0.5% w/w of emulgator D and free cells. After 6 hours in these conditions, the final viable cell numbers decreased to 5 log CFU mL$^{-1}$ for both capsules, while no viable cells were found when using free cells. It is possible to conclude that capsules have an important protective effect on *L. casei* cells.

Freshly prepared capsules and nude cells were also stored for 4 to 5 weeks that were mixed in milk, yogurt and white brined cheese. During these periods an inspection showed that both capsules and free cells were stable, proving that capsules do not play an important storage protection role in these dairy products.

Finally, some studies were performed to evaluate if the process could be scaled-up but more studies are required before a final conclusion can be retrieved.

**Key-words:** *Lactobacillus casei* LAFTI L26; emulsion encapsulation; gastrointestinal simulated conditions; capsules stability.
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<tr>
<td>$a_w$</td>
<td>Water Activity</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
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<tr>
<td>ME</td>
<td>Microencapsulation</td>
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<tr>
<td>MRS</td>
<td>De Man, Rogosa and Sharpe culture media</td>
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<tr>
<td>Re</td>
<td>Reynolds number of the stirrer</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>WCM</td>
<td>Whey cheese matrix</td>
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<td>WHO</td>
<td>World Health Organization</td>
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1. Introduction

1.1. Goal of the project

The initial goal of this project was to produce probiotic capsules with the desirable shape (spherical) and size (less than 100 µm) and to study their stability in different conditions. To achieve this goal it was necessary to optimize the encapsulation method by experimenting different emulsion compositions (emulsifiers and their concentration) and emulsification conditions (type of stirrers and stirring speed). After optimizing the encapsulation process the next step was to study the encapsulated cell’s stability upon storage in different environments and under human simulated GIT conditions compared to the stability of free cells. Finally, the capsules were evaluated and the viability of the inner immobilized cells incorporated into yogurt and their stability was compared with that of the free cells.

1.2. Starting point

Since the last year the research group of the Department of Diary, Fat and Cosmetics Science of Institute of Chemical Technology in Prague, has been working with encapsulation of probiotics. Earlier studies of microencapsulation have been performed with the Heidebach’s method and with extrusion methods with the use of calcium alginate as an encapsulation matrix. The group has decided to pursue the objectives but with the use of Heidebach’s encapsulation method because it is simple and uses milk proteins as encapsulation matrix which is better for the delivery of probiotics in milk products. The group also made some preliminary studies to determine the proper concentration of added lecithin to improve the emulsion.

1.3. Probiotics

1.3.1. Definition, regulation and guidelines

Probiotics can be defined as live microorganisms which, when administrated in adequate numbers, confer a health benefit on the host by improving its microbial balance. The benefit provided by probiotics are strain-specific which means that there is a link between the used strain and the health benefit provided. For this reason it is necessary to characterize the genus and strain of a microorganism when it is associated to a new health benefit to the host according with legal guidelines (Isolauri, et al., 2004).
The viability of probiotics in a product at the point of consumption is very important to correlate the administration dose with their efficiency. The lower limit that should be respected for the presence of probiotics in food is usually established by legal authorities as WHO and FAO. Also, when a new probiotic strain is being tested for use in foods, viability test to gastrointestinal conditions and to conditions of processing and storage should be performed. Probiotics must be metabolically stable and active in the product so they can be successful in the passage through upper digestive tract and then in adhering and colonizing the intestine system. Their survival in food and in the human body depends on the pH, post-acidification in fermented products during storage, hydrogen peroxide production, oxygen toxicity, storage temperatures and stability of the dried and frozen form, amongst others (Vinderola & Reinheimer, 2003).

Such as any new pharmaceutical substance needs approval from the official regulators, probiotics need to be recognized as safe and health benefit providers. For that reason, the FAO/WHO working group elaborated a report that summarizes the guidelines and regulations that probiotics must follow. The report (FAO/WHO Working Group, 2002) states that it is always necessary to identify the genus and species of a certain strain since health benefits are strain-specific. This identification should always be done with the use of proper taxonomy and methodology, and the FAO/WHO also recommends the elaboration of some genetic and phenotypic tests so the strain can be well characterized.

Probiotics can be responsible for some side-effects like systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer. Regarding these possible effects, *in vitro* tests should be performed and associated with *in vivo* results. Therefore, the tests that FAO/WHO recommends are: determination of antibiotic resistance; assessment of certain metabolic activities; assessment of side-effects during human studies; epidemiological surveillance of adverse incidents in consumers; if the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production; if the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.

Also, the strain must be clearly identified in the label of the product to inform the consumer, and labels should also contain the minimum viable concentration at the end of the shelf-life, the health claim, the proper storage conditions and the company contacts.

The recommendations indicated by the FAO/WHO working group are (FAO/WHO Working Group, 2002):

I. Adoption of the definition of probiotics as “Live microorganisms which when administrated in adequate amounts confer a health benefit to the host”;

II. Use and adoption of the guidelines in the report as a prerequisite for calling a bacterial strain “probiotic”;

III. Regulatory framework to allow specific health claims on probiotic food labels, just in cases where scientific evidences exists and to set the guidelines in the mentioned report;

IV. Promotion of these guidelines at an international level;

V. Good manufacturing practices (GMP) must be applied in the manufacture of probiotic foods with quality assurance, and shelf-life conditions established;
VI. Further development of methods (*in vitro* and *in vivo*) to evaluate the functionality and safety of probiotics.

1.3.2. Health benefits and applications

Probiotics have been indicated to have several health benefits, some of these have been well documented and established while others have shown a promising potential in animal models but still require human studies to substantiate its claims. Health benefits imparted by probiotic bacteria are always strain specific which means that there is no universal strain that provide all proposed benefits. The health benefits associated with probiotic strains that are already scientifically established are alleviation of lactose intolerance, prevention and reduction of symptoms of rotavirus and antibiotic associated diarrhoea. Probiotics have been indicated as potentially responsible for treatment and prevention of allergy, reduction of risk associated with mutagenicity and carcinogenicity, hypocholesterolemic effect, inhibition of *Helicobacter pylori* and intestinal pathogens, prevention of inflammatory bowel diseases and stimulation of immune system (Vasiljevic & Shah, 2008).

Lactose intolerance is caused by small activity of intestinal lactase (β-galactosidase) that causes insufficient lactose digestion in the small intestine, characterized by an increase in blood glucose concentration or hydrogen concentration in breath upon ingestion of lactose. The clinical symptoms of lactose malabsorption are bloating, flatulence, nausea, abdominal pain and diarrhea. These symptoms are caused by indigested lactose in the large intestine, where lactose is fermented by intestinal microflora. Probiotics can reduce lactose intolerance because intracellular β-galactosidase is expressed in the traditional cultures used in dairy fermentations that use lactose as energy source during growth, thus reducing its content in fermented products. Probiotics also increase β-galactosidase levels which is responsible for hydrolysing lactose (Vasiljevic & Shah, 2008).

One of the main applications of probiotics has been the treatment and prevention of rotavirus and antibiotic-associated diarrhoea, which is often caused by *C. difficile* after an antibiotic treatment. The mechanisms by which fermented dairy foods containing probiotics reduce the duration of diarrhoea are still unknown but one of the possible explanations is a competitive exclusion mechanism in which probiotics inhibit the adhesion of rotavirus by modifying the glycosylation state of the receptor in epithelial cells via excreted soluble factors. The presence of probiotics also prevents the disruption of the cytoskeletal proteins in the epithelial cells caused by pathogens which lead to the improvement of the mucosal barrier function and failure prevention in the secretion of electrolytes. Additionally, probiotic strains may modulate the innate immune response both to anti-inflammatory and pro-inflammatory directions (Vasiljevic & Shah, 2008).

There are some evidences that probiotic can prevent allergies. The mechanism of protective effects of probiotics on allergic reactions is not entirely known but it has been suggested that the reinforcement of the different lines of gut defence including immune exclusion, immune elimination and immune regulation could be the causes. Some studies have shown the ability of lactobacilli and bifidobacteria to decrease the genotoxicity activity of certain chemical compounds and increase antimutagenic activity during growth in selected media. Some of the possible explanations for these effects are microbial binding of mutagens to the cell surface, alteration of intestinal microecology and intestinal
metabolic activity, normalization of intestinal permeability and enhanced intestinal immunity. Also, it has been reported a potential anticarcinogenicity effect of probiotics that can be explained by the inhibition of enzymes responsible for the activation of procarcinogens. Another potential health benefit of probiotics is the hypocholesterolemic effect. There are two mechanisms that could be responsible for this effect. One explanation is the production of hydroxymethyl-glutarate by probiotic bacteria which was reported to inhibit hydroxymethylglutaryl-CoA reductases required for the synthesis of cholesterol. Another possible mechanism is the deconjugation of bile by bile salt hydrolase and co-precipitation of cholesterol with the deconjugated bile. The cholesterol is excreted via the faecal route and prior to its secretion the deconjugation of bile results in free bile salts. They are less efficiently absorbed and thus excreted in larger amounts in faeces. This effect is additionally augmented by poor solubilisation of lipids by free bile salts, which limits their absorption in the gut leading to further decrease of serum lipid concentration. This possible health benefit of probiotics still requires controlled human clinical trials to be established (Vasiljevic & Shah, 2008).

Probiotic cultures are capable to produce a wide range of antibacterial compounds like organic acids, hydrogen peroxide, bacteriocins, various low-molecular mass peptides, antifungal peptides/proteins, fatty acids, phenyllactic acid and hydroxyphenyllactic acid. Lactic and acetic acids are the main organic acids produced during the probiotic growth and they are responsible for lowering pH in the gastro-intestinal tract (GIT) which has a bacteriocidal or bacteriostatic effect. Bacteriocins are ribosomally synthetized antimicrobial peptides that are effective against other bacteria, either in the same species or across genera, although the producing strains are immune to their own bacteriocins. Thus probiotics can be responsible for preventing or reducing the detrimental effect of intestinal pathogens that compete for limited nutrients, inhibition of epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, production of antimicrobial compounds and the stimulation of mucosal immunity (Vasiljevic & Shah, 2008).

Inflammatory bowel disease includes a wide range of symptoms like inflammation, ulceration and abnormal narrowing of the GIT tract resulting in abdominal pain, diarrhoea and GIT bleeding. Several in vitro studies on cell models have shown the ability of certain probiotics to modulate the immune system and cause a beneficial action against inflammatory bowel disease (Guarner, et al., 2009).

To provide all these health benefits probiotics are used as food complement in form of capsules or as ingredients and usually applied in dairy products as yogurts, ice-cream, frozen desserts and cheese. Also, to be effective probiotics need to be present at a minimum concentration in the product during its shelf-life. Yogurts are thus the most popular product to contain probiotics although there are some parameters affecting bacteria viability. For instance, the product acidity, the acid production during storage (post-acidification), the oxygen level, the package permeability to oxygen, the susceptibility to antimicrobial substances produced by bacteria and the lack of some nutrients in milk are very important factors that may lead to significant loss of probiotic activity during storage (Kailasapathy K., 2002).

Another food that seems very promising to contain probiotics is cheese. Cheeses have many advantages over yogurt because they have a higher pH, a solid matrix and a high fat content which provides protection to the cells during storage and through GIT (Mirzaei, et al., 2012).
1.3.3. Synbiotics

The synbiotics concept refers to a mixture of prebiotics and probiotics which have health benefits to the host and also improves the survival and implantation of probiotics in the human gut, stimulating their growth and/or activation. Prebiotics are non-digestible food ingredients that when consumed in the right amount should specifically enhance the growth and viability of probiotics. These compounds play an important role improving probiotics’ activity and viability in the human body. The most common prebiotics are inulin, fructo-oligosaccharides and galacto-oligosaccharides (Oelschlaeger, 2012).

1.3.4. Probiotic strains

The most used strains as probiotics and the most reported in the literature belong to *Bifidobacterium* and *Lactobacillus* genera. Nonetheless most of the studies in this century are focused on *Lactobacillus* because of past practice and also because of the reputation of bifidobacteria as being difficult to work with and maintain.

*Bifidobacterium* is a Gram-positive bacteria, nonsporeforming, nonmotile, usually catalase-negative and strictly anaerobic. They grow at pH values in the range 4.5 – 8.5 with the optimal growth between pH 6.0 – pH 7.0 and at temperature between 37°C - 41°C with the minimum and maximum growth temperatures between 25°C and 43°C, respectively. *Bifidobacterium* forms colonies on agar which are smooth, convex with entire edges, with white or cream color, with glistening or soft consistency and which closely resemble those of lactic acid bacteria. *Bifidobacterium* can have cells of various shapes like short, regular and thin rods amongst others. Presently, there are 29 species from this genus, 14 isolated from human sources, 12 from animal intestine tracts or rumen and 3 from honeybees. All the species associated to humans can ferment lactose which is an important characteristic when delivering these species in foods because it lessens the discomfort of lactose malabsorption. Despite most of the species are safe, not all of the bifidobacteria are considered as safe to use in food. For example isolates from *B. dentium* can be isolated from dental caries and are potentially pathogenic. However, even the less safe species are not highly infectious or virulent in comparison to many common bacterial pathogens. Bifidobacteria have been shown to have immunological, antibacterial and antitumor activity in animals even though they demonstrate low antigenicity compared to other intestinal bacteria. Fermented and non-fermented milks, buttermilk, yogurt, sour cream, dips and spreads, ice-cream, powdered milk, infant formula, cookies, fruit juices and frozen desserts are some of the products where it is possible to find bifidobacteria used as a probiotic supplement (Anal & Singh, 2007).

*Lactobacillus* is a heterogeneous group of Gram-positive bacteria. The cells are nonmotile rods with tendency to form chains and are nonsporeforming. *Lactobacillus* have a fermentative metabolism, are facultative anaerobes, their surface growth on solid media generally is enhanced by anaerobiosis, increased carbon dioxide and reduced oxygen pressure. Strictly aerobic conditions are commonly growth inhibitory. *Lactobacillus* growth temperature is between 2 and 53°C; with optimal growth at 30-40°C, they grow at acidic conditions with optimal pH values between 5.5 and 6.2. Usually, growth occurs since pH 5.0 or even less and the growth rate is reduced in neutral or initially alkaline conditions (Roginski, 2003). *Lactobacillus* can be found as nature inhabitants in dairy products, grain...
products, meat and fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage, sourdough, water, soil and sewage. Such as *Bifidobacterium* they are part of the normal flora in the mouth, intestinal tract and vagina of humans and many animals. *Lactobacillus* share with other lactic acid bacteria the potential to inhibit the growth of competing undesired microorganisms and thus to prevent food spoilage. Nowadays, there are 96 species of *Lactobacillus* and 16 subspecies reported and their pathogenicity is absent in all of them. Infections caused by *Lactobacillus* species are very rare and have been estimated to represent 0.05-0.48% of all cases of infective endocarditis and bacteremia. In the vast majority of these cases an underlying disease indicated a predisposition of the patients (Bergey's Manual of Systematic Bacteriology, 2001).

In the present work the chosen commercial probiotic strain was *Lactobacillus casei* Lafti L-26. *L. casei* is facultatively heterofermentative, cells are nonmotile rods (0.7-1.1 µm x 2.0-4.0 µm), which often have square ends and form chains. Some of the growth factor requirements that are essential are riboflavin, folic acid, calcium phantothenate and riacin (Robinson, 2000). *L. casei* can be found in food ecosystems like raw and fermented milk, cheese, raw sausage and meat, fresh and fermented vegetables and is very often used as an additive culture during the production of semi-hard cheeses. In what concerns to probiotics *L. casei* administration has been reported as positive in many animals. *L. casei* strains are effective in the treatment of rotavirus diarrhoea in infants and in antibiotic-associated or traveller’s diarrhoea. There are also some evidences that *L. casei* strains significantly decrease faecal and urinary mutagenicity as well as the level of enzymes that produce carcinogenic derivatives (Roginski, 2003).

1.4. Encapsulation

1.4.1. Definition and goals

The encapsulation of microbial cells is a physicochemical or mechanical process to retain the microbes within other material to produce particles with diameter between 1 to 1000 µm. The encapsulation process has several purposes in the food industry (e.g. controlling oxidative reactions, masking flavours, colours and odours) but the main purpose of probiotic encapsulation is to enhance their stability. Probiotics are protected by encapsulation because capsules protect cells from unfavourable environmental conditions and also allow a controlled release in a viable and metabolically active state in the intestine (Nazzaro, et al., 2011).

The encapsulation process can occur naturally in nature when bacterial cells grow and produce exopolysaccharides. The cells become entrapped in their own secretions which act like a protective structure/capsule, reducing the permeability through the capsule and protecting the cells against adverse environmental conditions (Heidebach, et al., 2010).

In the development of a successful encapsulation system for a target microorganism it is necessary to know the stability of the encapsulated cells, the properties of the encapsulation material and also if the delivery system is suitable for the final application. The encapsulation process of probiotics usually has two main challenges. The first is the size of the produced capsules that should be under 100 µm but it depends on the chosen encapsulation technique. The other challenge is overcoming the low
viability of probiotics in dairy products and also in the GIT of consumers. Usually, the encapsulation process takes place in three stages. The first stage is the incorporation of the bioactive component, in the second the microcapsules are prepared and the last step is the microcapsules stabilization. Therefore, probiotics microencapsulation (ME) has the purpose to enhance the survival of probiotic bacteria during processing, storage and in particularly, in gastric transit.

1.4.2. Encapsulation materials
To encapsulate viable bacterial cells it is necessary to have a gentle process and a nontoxic material. The most common materials are polysaccharides from seaweed (k-carrageen, alginate), plants (starch and derivates, arabic gum), bacteria (gellan, xantham) and animal proteins (milk, gelatin). Alginate is the material used more frequently for encapsulation of probiotics but milk proteins are also becoming popular and they are the encapsulation material used in this project.

Milk proteins are natural vehicles to deliver micronutrients, cellular building blocks and immune system components from mother to newborn. Milk proteins are widely available and are usually regarded as safe raw materials with high nutritional value, have good sensory properties and also many structural properties and functionalities which make them highly suitable as vehicles to deliver different types of bioactives.

Cow milk proteins may be obtained from the skim milk after centrifugal separation of the cream, in which the milk fat globule membrane proteins are not worthy, by isoelectric precipitation of the caseins at pH 4.6 or by rennet clotting during cheese production, to obtain the casein rich precipitate and the serum containing the whey proteins. Then, caseinates can be obtained by resolubilizing the acid casein precipitate by alkali addition to about pH 6.7 and drying. The whey proteins can be obtained from whey by ultrafiltration, and whey protein isolates by subsequent diafiltration or ion exchange and then drying (Livney, 2012).

Milk proteins can bind big variety of molecules and ions with different degrees of affinity and specificity. Most of the milk proteins have an amphiphilic structure responsible for excellent surface properties. They have the ability to absorb at oil-water interfaces and stabilize emulsions due to their structure, flexibility, state of aggregation, dependent on pH, ionic strength and temperature of the solution. These proteins may form a barrier against penetration of oxidizing or other deteriorating agents which is highly beneficial. By absorbing to the oil-water interface, or by binding, entrapping or coating, the proteins form a shield, which is essential for protecting the encapsulated bioactives. Furthermore, milk proteins also have excellent gelation properties (e.g. acid or rennet curd formation of caseins, heated/acid/cold induced gelation of whey proteins or of total milk proteins), and some of the gelation mechanisms have been applied for probiotics entrapment. Milk proteins have excellent buffering capacity which also provides good shielding especially for probiotic microorganisms, against the harsh acid stomach conditions. The structure of the matrix formed by the proteins, with or without additional components, may form a barrier against diffusion and escape of the encapsulated bioactives, on one hand, and against inward access of digestive enzymes, on the other hand. Milk proteins generally have a good digestive biodegradability that can be useful to program the release of the bioactive payload to occur at the desired target location along the gastrointestinal tract and to
promote its bioavailability. For all of these properties milk proteins seem to have great potential to be used as vehicles for bioactives (Livney, 2012).

Table 1.1 shows examples of other encapsulation materials and their main properties.

**Table 1.1- Examples of encapsulation materials with the origin and main properties (Burgain et al., 2011).**

<table>
<thead>
<tr>
<th>Material</th>
<th>Origin</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>Polysaccharide extracted from diverse species of algae</td>
<td>High mechanical stability; High porosity and tolerance against salts and quelant agents; Non-toxic, biocompatible, low cost; Beads are sensitive to acidic environment; Difficult to scale-up; Limits to the use of alginate in food.</td>
</tr>
<tr>
<td>Cellulose acetate phthalate (CAP)</td>
<td>Polymer derived from cellulose</td>
<td>Insoluble in pH equal or below 5; Soluble to pH equal or higher to 6; Physiologically inert.</td>
</tr>
<tr>
<td>Starch</td>
<td>Polysaccharide formed with a high amount of glucose units</td>
<td>Good adhesion; Good storage and transit in the upper GIT; Resistant against environmental stress conditions.</td>
</tr>
<tr>
<td>K-Carrageenan</td>
<td>Natural polysaccharide extracted from aquatic macroalgae</td>
<td>Cells addition require temperatures between 40°C and 50°C; Gel is very fragile and cannot support mechanical stress conditions.</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Polysaccharide formed by glucosamine units. Isolated from crustacean shells, insect cuticles and fungi membranes</td>
<td>Properties change according to the extraction source; Low cell viability.</td>
</tr>
<tr>
<td>Xantham gum</td>
<td>Microbial polysaccharide derived from <em>Xanthomonas campestris</em></td>
<td>High resistance against acidic conditions.</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Protein gum which forms a thermoreversible gel</td>
<td>Amphoteric substance</td>
</tr>
</tbody>
</table>
1.4.3. Encapsulation methods

Spray-drying

Spray-drying is the most used method for microencapsulation in food industry because it is economical, flexible and capable to produce good quality products. In this method the cells are dispersed in a polymer solution and then the mixture is atomized in a drying chamber, in which the solvent (water) is evaporated from the microdrops produced therein and the microcapsules are formed (see Figure 1.1).

The advantages of the spray-drying method are operation in continuous mode, production speed, low cost, high reproducibility, suitability for industrial applications, ease of scale-up and the possible use of equipment that already exist in food industry. The principal limitations of the method are a small application field and the use of high temperatures which are not compatible with bacterial survival (Burgain, et al., 2011).

![Figure 1.1 – Example of a possible configuration of spray-drying technique for microencapsulation (Burgain et al., 2011).](image)

Extrusion

This technique uses hydrocolloids (alginate, carrageenan) as encapsulation materials. In this method an emulsion core and coating material are projected through a nozzle at high pressure. Extrusion of polymer solutions is mainly reported at laboratory scale where simple devices, like syringes, can be used. If the droplet formation occurs in a controlled way the technique is known as prilling. This is usually done by pulsation of the jet or vibration of the nozzle. The use of coaxial flow or an electrostatic field is the other common technique to form droplets. In Figure 1.2 there is a schematic representation of this encapsulation method. This is a very simple and cheap method, a gentle operation that does not cause cellular damage and provides a high viability. Furthermore this method does not use harmful solvents and can be operated both in aerobic or anaerobic conditions. However,
extrusion is mainly reported to laboratorial scale because it is difficult to scale up due to slow beads formation (Kailasapathy K., 2006).

Figure 1.2 - Schematic representation of extrusion technique with simple needle droplet-generator, usually air driven (a), and pinning disk device (b). The probiotic cells in a hydrocolloid solution pass through the nozzle and free-fall into a hardening solution like calcium chloride (Burgain et al., 2011).

**Emulsification**

Emulsification involves the dispersion of an aqueous phase containing the bacterial cells and polymer suspension into an organic phase, such as oil, resulting in a water in oil emulsion. The dispersed aqueous droplets are hardened by cooling or by addition of gelling or crosslinking agents. After gelation, the capsules are portioned into water and washed to remove the oil (see Figure 1.3). There are several modifications to the classic emulsification approach to improve its results. For instance it is possible to use coatings (polymer solution for e.g.), milk proteins or emulsification with interfacial polymerization. The emulsification is an easy process to scale up and provides a high cell survival rate. With this method capsules can have a small average diameter nevertheless, a wide range of capsules sizes and shapes are obtained in one single preparation still, this process enables tailoring the average capsules size, by manipulation of the stirring speed and the water/oil volume ratio.

In this project the encapsulation method used was developed by Heidebach (Heidebach et al., 2009) and uses a water-oil emulsion with milk proteins. One of the key substances used in this encapsulation method is rennet which is a proteolytic enzyme complex mainly consisting of chymosin. Chymosin is capable to cleave the k-casein molecule protruding from the surface of the casein micelles. When a sufficient amount of k-casein is hydrolysed, the other forms of casein inside the
micelle that does not have the ability to stabilize the micellar structure, are released and with the addition of calcium ions, lead to an aggregation of the casein micelles to form the gel. When the renneting process occurs at low temperatures the k-casein is cleaved, but the micelles do not coagulate until the temperature is raised above 18°C, where the gel is formed instantaneously (Heidebach et al., 2009). The Heidebach’s method makes use of this temperature triggered effect. In the encapsulation process the cold-rennet milk protein concentrate containing probiotic cells is dispersed in cold oil, forming an water-in-oil emulsion. Subsequently, the temperature is quickly raised above 18°C to start the gelification, the dispersed droplets will be transformed in small gel beads, and then raised to 40°C to optimize the gelification properties. In the next step the microcapsules are separated from the oil by gentle centrifugation (Heidebach et al.,2009). This emulsion encapsulation method is represented with more details in Figure 2.1.

Figure 1.3 – Example of emulsification procedure. The cell polymer suspension (discontinuous phase) is added to a large volume of oil (continuous phase), the mixture is homogenized to form a water in oil suspension. After the homogenization, the watery droplets will form tiny gel particles by addition of calcium chloride (Burgain et al., 2011).

**Spray-coating**

In this method the cells to encapsulate are in the solid state and are kept in movement in a special vessel. The main advantage of the spray-coating method is the easy-scale up and the possibility to apply several coats but this is a technique that is hard to master (Burgain et al., 2011). In Figure 1.4, the size range of capsules obtained with different methods is compared.
1.5. Previous studies

A lot of studies have been made related to the encapsulation of probiotics and to encapsulated probiotics stability when added in food or in GIT simulated conditions. The size of the probiotics capsule is an important parameter because it affects the sensory properties of foods. The probiotic capsules usually vary their size between 1 to 1000 µm but they should have less than 100 µm to avoid being noticed in food by consumers. In encapsulation studies using the emulsion technique and rennet-gelation of milk proteins, the authors have achieved mostly spherical shaped capsules and with a volume-based median diameter of 68±5 µm (Heidebach et al., 2009). Studies made with extrusion encapsulation with calcium alginate and resistant starch using Lactobacillus acidophilus La5, beads with diameter between 50 and 80 µm were reported for the production of Iranian white brined cheese (Mirzaei et al., 2012).

In order to promote health benefits, probiotics should be viable and in high concentration when ingested. Besides being a normal inhabitant of the intestine, to have a beneficial action probiotics should be delivered in $10^6$ to $10^7$ CFU g$^{-1}$ of product and consumed at levels higher than 100g/day. Thus, studies about the microcapsules stability in foods during their storage and in simulated GIT conditions were made to estimate if probiotics would reach the intestine in the recommended minimum concentration, upon the ingestion of the foodstuffs under evaluation.

In studies made with Bifidobacterium breve R070 in whey protein-based microcapsules stored in yogurt for 28 days at 4°C, a gradual decrease in the pH value to 3.9 - 4.0 was observed. The initial bacteria concentration was around $10^6$ CFU mL$^{-1}$ for encapsulated cells and $10^7$ CFU mL$^{-1}$ for free cells. After the 28 days of refrigerated storage, the number of viable free cells decreased to about $10^3$ CFU g$^{-1}$ and encapsulated bacteria decreased to about $10^4$ CFU g$^{-1}$ of product (Picot & Lacroix, 2004). In other study with Lactobacillus acidophilus 547 stored in yogurt showed a decline of about 1 log cycle over a period of 4 weeks for encapsulated cells and 2 log cycles for free cells. Furthermore,
microencapsulated *L. casei* 01 cells also survived better than the respective free cells with a higher survivability by 1 log cycle (Krasaekoot et al., 2006), (Brinques & Ayub, 2011).

Ice-cream is also part of the possible probiotic food carriers due to its composition which includes milk proteins, lactose, fat as well as other compounds. Studies were made with ice-cream matrix carrying encapsulated *B. lactis* (Bb-12) and *L. casei* (Lc-01) stored for 180 days at -20°C. For *L. casei* the initial cell counts were 9.4x10^5 CFU g^-1 for free cells and 5.8x10^5 CFU g^-1 for encapsulated cells. After 30 days of storage, the cell number slightly dropped to 5.1x10^5 CFU g^-1 and 4.8x10^5 CFU g^-1 for free and encapsulated cells, respectively. After 60 days of storage, the cell counts were 2.2x10^5 CFU g^-1 and 3.1x10^5 CFU g^-1 for free and encapsulated cells. At the end of the 180 days the cell numbers dropped around 3.4 log cycles for free cells and 1.4 log cycles for the encapsulated bacteria (Homayouni et al., 2008).

Cheese is also a possibility for incorporation of probiotics and can provide certain benefits over yogurt-type products. The advantages of using cheese as probiotic carrier over yogurt are the higher pH, higher fat content and also higher total solids content of cheese. These characteristics may offer protection for the probiotic cells against the harsh gastrointestinal environment. Studies were made with Iranian white brined cheese containing free and encapsulated *Lactobacillus acidophilus* La5 stored for 182 days at 5°C. The initial viable cell counts in cheese were around 10^10 CFU g^-1 for free cells and around 10^15 CFU g^-1 for encapsulated cells. After 28 days of storage the number of free cells increased to 10^12 CFU g^-1 and the number of encapsulated cells remained sensively constant. At the end of the storage period the cell counts were 10^5 CFU g^-1 for free cells and around 10^11 CFU g^-1 for encapsulated cells (Mirzaei et al., 2012).

In what concerns to GIT conditions it is important to see how capsules react to the harsh stomach conditions and also to intestine conditions. In simulated gastric juice at 37°C, cells of *Bifidobacterium breve* R070 with the initial concentration of around 10^6 CFU mL^-1 whether free or encapsulated, the free cells showed 6 log reduction after 30 minutes at pH 1.9. Nevertheless, the number of viable cells remained constant for the next 60 minutes (10^6 to 10^3 CFU mL^-1) and increased significantly after 3 and 6 hours to about 10^5 CFU mL^-1 at pH 7.5. For encapsulated cells, the viable cell counts were 1.9x10^7 CFU mL^-1 after 1 hour incubation in GIT conditions and increased to 7.2x10^6 CFU mL^-1 after 6 hours in the presence of pancreatin and bile salts, respectively. These numbers indicate that the damage caused to the cells were only temporary due to low pH stress (Picot & Lacroix, 2004). Similar studies were made with *Lactobacillus rhamnosus* LC705 with an initial concentration around 10^6 CFU mL^-1 for free and encapsulated cells (95.9% of encapsulation efficiency). The number of viable free cells in low pH decreased from 10^6 CFU mL^-1 to 10^4 CFU mL^-1 after 2 hours (28.5% less) and in bile salt conditions it increased to 10^6 CFU mL^-1. When encapsulated, the number of viable cells increased from 10^6 CFU mL^-1 to 10^7 CFU mL^-1 (9.2% more) and in bile salts it increased to 10^6 CFU mL^-1 (28.5% more) (Pimentel-González et al., 2009). In a study with *Lactobacillus casei* LAFTI L26, the probiotic strain was present at levels of 10^7 to 10^9 CFU g^-1 at the beginning of digestion for free and encapsulated cells, as classically recommended. Free cells of *L. casei* decreased its viable cell numbers by 1 log cycle when exposed to mouth conditions, when pH reached 2.8 in the presence of pepsin, the cells suffered a slight decrease in viable numbers and after 92 minutes, in duodenum
conditions, the probiotic strain was practically vanished. For encapsulated *L. casei* in whey cheese matrix, the mouth conditions did not affect the cells. During passage through the oesophagus-stomach step, a decrease of 1.5 log cycles was observed and pH reached only 3.8. In the simulated duodenum conditions *L. casei* even increased 1 log cycle in viable numbers. Thus, the viable cell counts for encapsulated cells decreased overall 2 log cycles, relative to the starting situation (Madureira et al., 2010).
2. Methods

2.1. Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Balance</td>
<td>6110 Balance, Tecator (Sweden)</td>
</tr>
<tr>
<td>CO₂ Incubator</td>
<td>Sanyo Incubator MCO-17AIC, Schoeller (Japan)</td>
</tr>
<tr>
<td>Fluorescent Microscope</td>
<td>BX-51 Olympus Microscope - C-5050 zoom Olympus Camera (Japan)</td>
</tr>
<tr>
<td>Halogen Moisture Analyzer</td>
<td>HR73, Mettler Toledo (Switzerland)</td>
</tr>
<tr>
<td>Incubator</td>
<td>BMT MMM-Group, Ecocell (Germany)</td>
</tr>
<tr>
<td>Magnetic Stirring Hotplate</td>
<td>MR Hei-Tec, Heidolph (Germany)</td>
</tr>
<tr>
<td>Mastersizer</td>
<td>Mastersizer 2000, Hydro 2000 S, Malvern instruments (United Kingdom)</td>
</tr>
<tr>
<td>Optical Microscope</td>
<td>Leica DMLS - DFC320 Leica Camera (Germany)</td>
</tr>
<tr>
<td>pH Meter</td>
<td>pHnomenal ™, VWR International (Germany)</td>
</tr>
<tr>
<td>Precision Balance</td>
<td>Precisa Balances (Zurich, Switzerland)</td>
</tr>
<tr>
<td>Reflectometer</td>
<td>RQflex®plus 10 Reflectoquant®, Merck Milipore (Germany)</td>
</tr>
<tr>
<td>Small Centrifuge</td>
<td>D-78532 Tuttligen, Hettich (Germany)</td>
</tr>
<tr>
<td>Temperature Sensor</td>
<td>EKT Heicon®, Heidolph(Germany)</td>
</tr>
<tr>
<td>Vortex</td>
<td>Yellowline TTS2, IKA® Works Inc (USA)</td>
</tr>
<tr>
<td>Water Activity Meter</td>
<td>Aqua Lab CX3, Decagon (USA)</td>
</tr>
</tbody>
</table>

For the scale up experiment, the vessel had a volume of 4.75 L and maximum working volume of 3.5 L. The stirrer had 2 perpendicular impellers with 5 cm of diameter and 3 holes with 1 cm of diameter. The stirrer has a maximum torque of 400 Ncm, viscosity up to 60 Pa.s and stirring capacity up to 25 L of water. The setup of the process is represented in section 3.6 in Figure 3.10.
2.2. Materials

Solutions

Physiological solution
Peptone (1 g) and NaCl (8.5 g) were dissolved in distilled water. The pH was adjusted at 7.0. The peptone used was bacteriological peptone produced by OXOID LTD. (Hampshire, England), with 14.0% (w/w) of total nitrogen, 2.6% (w/w) of amino nitrogen and 1.6% (w/w) of sodium chloride.

Gastrointestinal solution
For stomach simulation NaCl 0.5% (w/v) was dissolved in distilled water and the pH adjusted to 2.0 - 2.1. After sterilization 0.3% (w/v) pepsin was added to final concentration. After 2 hours, to simulate intestine conditions, 0.3% (w/v) bile salts and 0.1% (w/v) pancreatin were added and the pH was adjusted to 7.0. The pepsin was from hog stomach with 1436 U/mg and produced by Sigma-Aldrich (Switzerland). The pancreatin was obtained from hog pancreas with 165 U/mg of amylase activity, produced by Sigma-Aldrich (Switzerland). The ox bile salts used were tauroglycocholic acid sodium salt (bile salt content >65%), produced by Merck (Germany).

CaCl₂ solution
CaCl₂ was prepared at 10% (w/w) with distilled water. The calcium chloride (anhydrous powder) was produced by Lach-Ner (Czech Republic) with molecular weight of 110.99g/mol, with minimum assay of CaCl₂ of 96% and maximum of MgCl₂ of 1.5%.

Rennet Naturen® Premium 145
Composed by water, chymosin, pepsin, baking soda, sodium benzoate E211 (less than 1%), minimum activity of 145 IMCU/ 1 mL, produced by CHR Hansen.

Duolac buffer
\[ \text{Na}_2\text{HPO}_4 \ 0.60\% \ (w/v), \ 0.45\% \ (w/v) \ \text{KH}_2\text{PO}_4, \ 0.05\% \ (w/v) \ L\text{-cystein HCl}, \ 0.05\% \ (w/v) \ \text{Tween 80} \]
were dissolved in distilled water and the pH was adjusted to 5.8 (Cell Biotech).

Citrate buffer
The citrate buffer was prepared with 20g of natrium citrate dehidrated, dissolved in 1 L of distilled water. After sterilization the pH was 7.5.

Reconstituted Skim milk
Reconstituted skim milk 35% (w/w) was prepared from dehydrated milk by dissolving in destilated water. This solution was not sterilized. The reconstituted skim milk used was produced by PML Protein Mlėko Laktóza, a.s. (Czech Republic).
Rhodamine B
For fluorescent microscopy the sample was stained with a solution of 0.2% (w/w) of rhodamine B dissolved in ethanol. The rhodamine B was produced by Sigma-Aldrich, with a dye content of 95%.

Canola oil
The oil used for encapsulation was regular cooking canola oil bought in a local store.

Growth Media

MRS agar

*Lactobacillus* agar, accordingly to De Man, Rogosa and Sharpe for microbiology, MERCK.
Composition:
10.0 g/L Peptone from casein
10.0 g/L Meat extract
4.0 g/L Yeast extract
20.0 g/L D (+) – Glucose
2.0 g/L di-Potassium hydrogen phosphate
1.0 g/L Tween 80°
2.0 g/L di-Ammonium-hydrogencitrate
5.0 g/L Sodium acetate
0.2 g/L Magnesium sulphate
0.04 g/L Manganese sulphate
14.0 g/L Agar-agar
For yogurt and cheese it was necessary to adjust pH value of MRS to 6.2 and add vancomycin to a final concentration of 10 µg/mL. Vancomycin inhibits the starter cultures and allows the selective enumeration of LAFTI® L26 as described in DSM protocol.

MRS broth

*Lactobacillus* agar, accordingly to De Man, Rogosa and Sharpe for microbiology, MERCK.
Composition:
10.0 g/L Peptone from casein
8.0 g/L Meat extract
4.0 g/L Yeast extract
2.0 g/L di-Potassium hydrogen phosphate
1.0 g/L Tween 80°
2.0 g/L di-Ammonium-hydrogencitrate
5.0 g/L Sodium acetate
0.2 g/L Magnesium sulphate
0.04 g/L Manganese sulphate
Emulsifiers

Lecithin

Lecithin was produced by Solae Europe S.A. (Geneva, Switzerland) with purity of 96%.

Emulgator D

Emulgator D was produced by Danisco Ingredients with a content of fatty acids of 30.2% of C16:0, 2.3% of C16:1, 19.9% of C18:0, 34.6% of C18:1, 9.2% of C18:2 and 3.8% of others and content of free fatty acids of 0.4% of FFA, 87.8% of MAG, 3.3% of DAG and 8.5% of TAG.

2.3. Procedures

2.3.1 Experimental procedures

Cell culture

To obtain fresh culture of *L. casei* for the production of capsules, lyophilised culture stored in the freezer was cultured in MRS broth 2% v/v of inoculum, with pH 5.6 and during the night at 37°C and 5% of CO₂.

Encapsulation

The encapsulation process was based on the method described by Heidebach and co-authors (2009) (see Figure 2.1). Thirty Grams of skim milk solution 35% (w/w) were prepared with sterile distilled water and kept at 5°C during two hours with magnetic agitation in a closed 50 mL vessel. Afterwards, about 2 g of fresh culture were added to the skim milk solution and 400 µl of diluted rennet (1:5). The solution was then incubated for 60 minutes at 5°C with agitation. After incubation, 180 µL of 10% (w/v) CaCl₂ were added to the skim milk solution. Immediately, 15 g of this milk solution were added to 150 mL of oil at 5°C, stirred for 5 minutes to emulsify the mixture into the oil. Then, the mixture was magnetic stirred and heated until 40°C and the stirring and temperature maintained for more 15 minutes. After the encapsulation process it was necessary to separate the capsules from the oil by gentle centrifugation (500 g, 2 minutes), the supernatant was removed, distilled sterile water was added and the sample shaken a few times. To determine the appropriate number of such washing steps, several washings were performed and samples were collected from supernatant and sediment for bacteria counting (see Table 2.2 ).
Table 2.2 – Bacteria cell counting for supernatant and sediment in several washings of capsules to determine the appropriate number of washings.

<table>
<thead>
<tr>
<th>Number of washings</th>
<th>Supernatant (CFU/mL)</th>
<th>Sediment (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.66x10^5</td>
<td>5.41x10^7</td>
</tr>
<tr>
<td>3</td>
<td>1.77x10^5</td>
<td>3.55x10^7</td>
</tr>
<tr>
<td>5</td>
<td>1.72x10^5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>8.5x10^4</td>
<td>4.09x10^7</td>
</tr>
</tbody>
</table>

After the bacterial counting it is possible to see that the values of capsules present in the supernatant and sediment for the 1st, 3rd, 5th and 6th was similar which means that 3 washing times would be enough.

During this study different emulsifiers were added to the oil, in different concentrations, to understand their influence in capsules size. Also the agitation speed during the temperature increase from 5°C to 40°C was altered from 500 rpm and 1000 rpm to understand the effect of agitation in capsules size.

The encapsulation procedure is represented in Figure 2.1.

Figure 2.1 – Microencapsulation process developed by Thomas Heidebach and co-workers by means of rennet-induced gelation (Burgain et al., 2011).
**GIT Simulation**

For the experiment in simulated gastrointestinal conditions freshly cultured free cells, capsules with 0.5% w/w of lecithin and capsules with 0.5% w/w of emulgator D were tested. The sample of free cells or capsules was dissolved in the gastrointestinal solution described previously and samples of 1 mL were collected at 0, 2, 4 and 6 hours of incubation. The first two hours of this experiment represents stomach conditions and after two hours the solution simulates small-intestine conditions. After collecting the sample of free cells, several dilutions were made in physiological solution and sample was inoculated in MRS agar plates for bacteria counting. The sample with capsules was first diluted in citrate buffer to release the cells, according with the procedure that is described in the analytical procedures section, and after all the dilutions were made in physiological solution and inoculate for bacteria counting.

**Storage Experiment**

The goal of this experiment was to access the capsules stability when stored in different types of food. The capsules were stored in milk, yogurt and cheese. In this experiment, in each different type of food, free cells and capsules produced with 0.5% w/w of lecithin were stored. For milk and yogurt the samples were stored for 6 weeks at 4°C, once a week 1 g of sample was collected for bacteria counting and pH measurement and after, the samples with 100 mL were kept in the fridge and were not sealed. The starter culture used to produce yogurt was YC-381 from CHR Hansen (Denmark), with 2% of inoculum and fermented for 4 hours at 40°C. The milk for storage in milk and yogurt samples was prepared with 10 g of reconstituted skim milk with 1.5% fat content and 90 ml of water, the milk was sterilized at 100°C for 20 min.

The cheese was produced with 3 L of pasteurized milk at 72°C for 20 s, with 3.5% of fat content and 3.1% of protein content. To produce the cheese 4% of starter culture was used from Milcom a.s., Laktoflora®, containing the microorganisms *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *diacetilactis*. For renneting 15 mL of the commercial enzyme formulation (dilution 1:9) were added at 33°C and after being made, the cheeses were kept in a brine solution 16% (w/w) for 45 minutes. The cheeses were stored for 4 weeks in the brine solution (ratio cheese:brine 1:1 w/w) at 8°C, approximately. Once a week, and for up to 4 weeks, one cheese with free cells and another with capsules were collected to measure water activity, dry-matter content, pH and bacteria counting. For the milk and yogurt the samples had 5 g of capsules and for cheese around 25 g of capsules were added. To estimate the free cell concentration it was expected that in rich media as MRS, lactobacilli could grow up to $10^{10}$ - $10^{11}$ CFU/mL and, by knowing the volume of media, it was possible to calculate the appropriate dilutions to have the cell concentration of $10^8$ CFU/mL or g, in the beginning of the experiment. Free cells and capsules were added in pellet and dissolved in milk solution or in yogurt.
2.3.2 Analytical procedures

Bacteria Counting

To estimate the number of viable cells, a method that relies on growth of bacterial cells in agar plates was used. A sample of 1g (solid sample) or 1 mL (liquid sample) was collected and diluted in 9 mL of physiological solution. Then a serial of dilutions were prepared depending on the expected CFU in the initial sample. Two appropriate dilutions were chosen for sample incubation in MRS agar for 48 hours at 37°C; two Petri dishes were used for each dilution. After the incubation period, the colonies formed on each plate were counted. Only the plates with 30 to 300 colonies in and on the agar were considered for the study, to have a good statistical representation of the number of bacteria in the undiluted sample. The concentration of microorganisms was calculated according to Equation 1. In this method it is assumed that all viable cells evolved to colonies and that each colony counted is formed from just one bacterial cell.

\[
C = \frac{\sum N}{V \times (n_1 + 0.1 \times n_2) \times d} \quad \text{Equation 1}
\]

Where,

- \(C\) – Number of microorganisms in 1 g or 1 mL of sample (CFU/g or CFU/mL);
- \(\sum N\) – Total number of colonies from all plates used;
- \(V\) – Volume of sample inoculated on the plate (mL);
- \(n_1\) – Number of plates of the first dilution used;
- \(n_2\) – Number of plates of the second serial dilution used;
- \(d\) – Dilution factor of the first dilution used.

Microscope Preparations

The microscope preparations for optical microscopy were in native state, colored Gram staining, methylene blue or sudan. In the native form, the sample was suspended in a drop of water. The Gram staining was used to differentiate Gram-positive and Gram-negative bacteria, the methylene blue was used to dye the bacteria among proteins in the matrix, sudan stain was used to color the lipids (Society of American Bacteriologists, 1957). All the preparations, except for the native preparation, were fixed by flame.

For fluorescence microscopy the sample was stained with rhodamine B. Rhodamine B is xanthene derivative and it may be regarded as fluorescein in which the two hydroxyls are replaced by diethylamino groups (Lundgren & Binkley, 1954). In aqueous solution rhodamine B is red over a wide
pH range but in strong acid conditions the dye becomes orange and in strong alkali conditions blue. To stain the capsules rhodamine B was dissolved in alcohol which has an excitation wave length of 554 nm and emission wave length of 627 nm (Prahil, 2013). The fluorescent microscope had an excitation and emission wave length of 450 – 510 nm and 600 – 660 nm, respectively.

**Mastersizer**
Laser diffraction, based on the optical properties of dispersion was used to determine the size of the microcapsules. The capsules were re-suspended in demineralised water with refractive index 1.33 and the mean size distribution was measured using Mastersizer 2000 (Malvern, Worcestershire, UK) with the dispersion unit Hydro G (Malvern, Worcestershire, UK). A refractive index 1.45 and absorption coefficient of 0.001 was selected for a sample of microcapsules. The measurement was performed at 25°C. The Mastersizer uses the technique of laser diffraction to measure the size of particles by measuring the intensity of light scattered as a laser beam passes through a dispersed particulate sample. This data is then analysed to calculate the size of the particles that created the scattering pattern. The results are the means from 2 independent encapsulation procedures, encapsulated cell samples were analysed seven times. The mean of volume based median diameter (d$_{0.5}$) (i.e. 50 % of total volume is composed of microcapsules with diameters equal or lower than d$_{0.5}$), and 90 % fractiles (d$_{0.9}$) were calculated. For the used equipment, the particle size range to be measured must be between 0.02 and 2000 µm. In this case, measurements have an accuracy better than 1% (polydisperse standard) and a reproducibility better than 1% variation (polydisperse standard).

**Bacterial Release from Capsules**
To estimate the number of viable cells in a sample with microcapsules it was necessary to release the cells from inside the capsules. Two different buffers were experimented to determine which one was more efficient. For citrate buffer, the samples (1 mL or 1 g) were diluted first in 9 mL of the buffer, vortexed for 5 min, allowed to rest for another 5 min, and the process repeated during 20 min. Then, dilutions were made with physiological solution following the method used for bacterial counting that was previously described. For Duolac buffer, 1 g of the sample was dissolved in 9 mL of the sterilized diluent and vortexed for 2 min followed by 3 min resting and the process repeated during 20 minutes. Afterwards, the subsequent dilutions were made in physiological solution as described above in the bacteria counting method.

**Dry Matter Content**
To measure the dry matter content a weighed test portion was mixed with sand and dried by heating it in a drying oven at 102°C until constant sample weight. Then, the weight loss is calculated. This procedure follows the method described in ISO 5534:2004.
**Water Activity**

The water activity ($a_w$) indicates how tightly the water is bound, structurally or chemically, within a substance and is an important factor to control the rate of deterioration. A higher $a_w$ value means that the water has a higher motility so is more available inside the food to react or facilitate reactions, as a solvent. The general lower limit for bacterial growth is $a_w = 0.90$. Almost all microbial activity is inhibited below $a_w = 0.6$, most fungi are inhibited below $a_w = 0.7$, most yeasts are inhibited below $a_w=0.8$ and most bacteria below $a_w = 0.9$ (Fellows, 1988).

The water activity was measured in Aqua Lab CX3. This device uses the chilled-mirror dewpoint technique to measure the $a_w$ of the sample. The sample is equilibrated with the headspace of a sealed chamber that contains a cold mirror and a sensor to detect the formation of the first droplets in the mirror. After knowing this temperature, the $a_w$ is calculated because, at equilibrium, the relative humidity of the air in the chamber is the same as the $a_w$ of the sample.

**Lactic Acid Concentration**

The lactic acid concentration in MRS broth during bacterial growth is a measurement of the metabolic activity of lactic acid bacteria. The lactic acid concentration was measured in RQflex® plus 10 Reflectoquant® with specific test strips for lactic acid. In this device the lactic acid is oxidized by nicotinamide adenine dinucleotide (NAD) under the catalytic effect of lactate dehydrogenase to a pyruvate. In the presence of diaphorase, the NADH formed in the process reduces a tetrazolium salt to a blue formazan that is determined reflectometrically. The measuring range of this device was 3.0 mg/L to 60.0 mg/L of lactic acid.

**Reynolds Number of Stirrer**

To scale-up the encapsulation process the agitation speed is a very important parameter related with the power that is absorbed by the system. Since it was not possible to calculate the agitation power in an accurate way, the Reynolds coefficient was calculated as an indicator parameter. To calculate the agitation power is necessary to calculate the Reynolds number of the stirrer. The Reynolds number was calculated according to Equation 2 and 3.
\[ Re = \frac{f \phi_{\text{stirrer}}^2}{v} \]  \hspace{1cm} \text{Equation 2}

\[ v = \frac{\mu}{\rho} \]  \hspace{1cm} \text{Equation 3}

Where,

- \( f \) - agitation speed (s\(^{-1}\));
- \( \phi_{\text{stirrer}} \) - stirrer diameter (m);
- \( v \) - kinematic viscosity (m\(^2\)/s);
- \( \mu \) - viscosity (Pa.s);
- \( \rho \) - density (kg/m\(^3\));

The Re was calculated for the emulsion considering only the oil viscosity at 5°C and 40°C and the stirring speed of 500 rpm. The oil density and viscosity were measured for different temperatures. For 5°C the Re = 130 which means that the emulsification process occurred under laminar flow regimen. For 40°C the Re = 587 which means that the flow reached a transient regimen, but not fully developed turbulent flow. In order to calculate the Re the density and the viscosity of the oil were measured. The density of the oil was measured by weighing an exact volume of oil at certain temperature. The viscosity was determined on rotary viscometer Kinexus (Malvern Instruments, Malvern, UK) in the coaxial cylinders geometry C25/PC25 DIN. After the filling of geometry, the sample was tempered to the selected temperature (5 – 45°C), for 5 min. The viscosity was determined at shear rate 100 s\(^{-1}\) for 100 sec, in interval 5 sec. The results are the mean of 20 values. All the calculations are detailed in Appendix 6.1.
3. Results and Discussion

The probiotic bacteria encapsulated was *L. casei*, at the beginning of the experiments they were observed at microscope and some pictures collected (see Figure 3.1) to confirm the morphology described in section 1.3.4.

![Figure 3.1- L. casei by Gram staining stained in Gram (left) and by methylene blue (right).](image)

3.1. Capsules production

In the encapsulation process the influence of different parameters was studied. Capsules were produced without any surfactant agent, produced with two different emulsifiers and different concentrations (4% w/w of lecithin, 0.5% w/w of lecithin and 0.5% of emulgator D, in oil) and two different agitation speeds (500 rpm and 1000 rpm). The goal was to see the influence of these parameters on the capsules size and partial size distribution and shape. The size and shape of the capsules are very important for protection effects but mainly because of foods sensory properties. In one hand, capsules should be spherical and the average size should be sufficiently small in order to avoid sensorial impact on the product. On the other hand, larger capsule diameters increase the likeliness of a protecting effect. So it is suggested a size below 100 µm as desirable to avoid negative sensorial impacts for microcapsules in food.

To find the best strategy for encapsulation, all the capsules produced at the same agitation speed where compared and the mean of volume based median diameter ($d_{50}$) were represented in Figure 3.2a and b. For capsules produced without emulsifier and with different concentrations of lecithin, the particle size distribution was measured by Mastersizer. For capsules produced with emulgator D it was
not possible to use Mastersizer because capsules formed aggregates that impossible to disperse in water neither with SDS. Therefore, for emulgator D 20 capsules of two independent productions were measured by optical microscopy and the average diameter was calculated.

For encapsulation at 500 rpm, it is possible to notice in Figure 3.2a that the biggest average diameter was obtained for capsules produced with no emulsifier as it would be expected. For all the other formulations the average diameter was below 100 µm as is recommended for microcapsules in food.

For capsules produced with 4% w/w of lecithin and 0.5% w/w of emulgator D, the volume based median diameter was not very different so we can conclude that emulgator D was a more effective emulsifier. The capsules with 4% w/w lecithin were the smallest but such high concentration of lecithin conveyed a brown colour to the capsules and that impacts negatively when planning to apply them in dairy products (see Figure 3.3). Capsules with emulgator D were also very small but they tended to form aggregates. Although capsules with 0.5% w/w of lecithin were bigger than 4% w/w of lecithin and 0.5% w/w of emulgator D, this condition was the best result because they had a volume based median diameter under 100 µm and they were white.

Figure 3.4 shows typical particle size distributions obtained in Mastersizer. The capsules with the biggest average size, no emulsifier, had the narrowest particle size distribution which means that the capsules diameter was more homogeneous. The production with higher concentration of lecithin had the smallest diameter but the widest distribution, i.e. the more heterogeneous distribution. This heterogeneous distribution is also a drawback for capsules with 4% w/w of lecithin.

For encapsulation at 1000 rpm, it is possible to see in Figure 3.2b, that the most significant different result obtained was for capsules without emulsifier. For this condition, at 1000 rpm, the volume based median diameter was smaller than 100 µm. For all the other conditions there were no significant changes so it is possible to conclude that, in the presence of an emulsifier agent, the agitation speed does not determine capsules size. In Table 3.1 are the values of the mean of volume based percentile d(0.9) for capsules measured in Mastersizer for 500 and 1000 rpm. Based in these values we can conclude that the higher d(0.9) are for capsules without emulsifier and for 500 rpm as expected. These results mean that speed has a higher influence in capsules average diameter when no emulsifier is used. Also the standard deviation is high for all the formulations, especially for the ones without emulsifier.

In Figure 3.4 it is represented the particle size distribution for 1000 rpm. The results obtained for this agitation speed were similar to 500 rpm. The narrowest particle size distribution was obtained for capsules without emulsifier and bigger diameter and the widest distribution was obtained for 4% w/w of lecithin, smallest diameter.
Figure 3.2 – Volume based median diameter ($d_{0.5}$) of capsules without emulsifier (red), 4% w/w of lecithin (green), 0.5% w/w of lecithin (purple) and 0.5% w/w of emulgator D (blue), the encapsulation was made at 500 rpm (A) and at 1000 rpm (B). The measurements for capsules without emulsifier and lecithin were performed by Mastersizer and each bar was calculated with 2 independent productions and 7 measurements for each. For emulgator D, about 20 capsules were measured from 2 different productions with optical microscope and the mean was calculated. The error bars represent the standard deviation.
Table 3.1 - Results of mean of volume based 90% fractiles ($d_{0.9}$) from mastersizer and standard deviation ($\sigma$) to capsules produced without emulgator, 4% w/w of lecithin, 0.5% w/w of lecithin at 500 and 1000 rpm.

<table>
<thead>
<tr>
<th>Emulsifier</th>
<th>Speed (rpm)</th>
<th>$d_{0.9}$ (µm)</th>
<th>$\sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>500</td>
<td>382.9</td>
<td>209.4</td>
</tr>
<tr>
<td>4% w/w Lecithin</td>
<td>500</td>
<td>56.4</td>
<td>21.5</td>
</tr>
<tr>
<td>0.5% w/w Lecithin</td>
<td>500</td>
<td>120.4</td>
<td>72.9</td>
</tr>
<tr>
<td>No</td>
<td>1000</td>
<td>228.4</td>
<td>60.7</td>
</tr>
<tr>
<td>4% w/w Lecithin</td>
<td>1000</td>
<td>47.6</td>
<td>14.3</td>
</tr>
<tr>
<td>0.5% w/w Lecithin</td>
<td>1000</td>
<td>141.3</td>
<td>20.7</td>
</tr>
</tbody>
</table>

Figure 3.3 – Image of bulk moist of capsules produced with 4% w/w of lecithin (a), 0.5% w/w of lecithin (b) and 0.5% w/w of emulgator D (c) in oil, at 500 rpm, after washing.
Figure 3.4 – Particle size distribution for encapsulation with no emulsifier (red), with 4% w/w of lecithin (green) and 0.5% w/w of lecithin (blue) in oil. All capsules were produced at 500 rpm (A) and at 1000 rpm (B).

Thomas Heidebach and co-workers (2009), using the same formulation and method with no emulsifier at 500 rpm obtained capsules with a volume based diameter of 68 µm. Fritzen-Freire et al. (2012) obtained capsules with an average diameter of 18.78 µm with spray drying.

After analysing the previous results, it was considered that the more satisfying result for encapsulation by emulsification gelification was obtained for 0.5% w/w of lecithin. Therefore, this composition was chosen to produce capsules for the subsequent experiments at 500 rpm since the influence of agitation speed was not significant.

Capsules Structure

In order to analyse the capsules structure and shape, several pictures were collected by optical microscopy, fluorescence microscopy and electronic microscopy. In optical microscopy, capsules were prepared by different methods as it is possible to see in Figure 3.5 for capsules prepared with 0.5% w/w of lecithin at 500 rpm. In Figure 3.5a the capsules were stained with methylene blue which marks the DNA and RNA and it permits the visualization of the L. casei cells inside the capsules. In Figure 3.5b the capsules were in their native form and in Figure 3.5c capsules were stained with sudan and it is possible to see a thin layer of oil around them.
Figure 3.5- Capsules produced with 0.5% w/w of lecithin in oil at 500 rpm and microscope preparations obtained by different methods. Capsules pictures were obtained when stained with methylene blue (1600x magnification) (a), in native form (640x magnification) (b) and stained with sudan (1600x magnification) (c). The scale bar in pictures a, b and c represent 20 µm.
For fluorescent microscopy capsules from a fresh sample were stained with rhodamine B which should mark the proteins in orange colour. In fluorescence microscopy it was expected to see capsules stained in orange because of the protein matrix, and with a black outside layer that would be due to the oil. In Figure 3.6 it is possible to see the capsules stained in orange as expected but the oil layer is not clear. One possible explanation for this result is an inappropriate sample preparation.

![Image of stained capsules](image)

**Figure 3.6 – Sample with capsules produced with 0.5% of lecithin at 500 rpm stained with rhodamine B and observed by fluorescent microscopy at 100x magnification (λ<sub>ex</sub>=554 nm; λ<sub>em</sub>=627 nm).**

### 3.2. Cell release

In order to count the cells entrapped inside the capsules it was necessary to release them before following the plating method for bacteria counting described in section 2.3.2. The cells were suspended in citrate buffer and duolac buffer and microscopy preparations were made after 10 and 20 minutes. Also, after 20 minutes, 1 mL of the suspension of sampled cells in buffer were collected for bacteria counting. The cell concentration after 20 minutes was 9.7 log CFU mL<sup>-1</sup> for citrate and duolac buffer. However, by analysing microscopy preparations after 10 and 20 minutes for both buffers (see Figure 3.7 and 3.8) it is possible to see that cell release appears to be more effective for citrate buffer since bigger particles remained intact in duolac buffer. Therefore it was decided to use citrate buffer to release the cells from capsules to perform the cell counting.
Figure 3.7 – Microscopy preparations of capsules stained with methylene blue. Capsules with 0.5% w/w lecithin were previously dissolved in citrate buffer to release the cells for bacterial counting. The cells were vortexed for 20 minutes with pulses (5 minutes vortex, 5 minutes resting) and preparations made at 10 minutes (right figure) and 20 minutes (left figure).

Figure 3.8 - Microscopy preparations of capsules stained with methylene blue. Capsules with 0.5% w/w lecithin were dissolved previously in Duolac buffer to release the cells for bacterial counting. The cells were vortexed for 20 minutes (2 minutes vortex, 3 minutes resting) and preparations made at 10 minutes (right figure) and 20 minutes (left figure).
3.3. Simulation of gastro-intestinal conditions

One of the main problems associated with probiotic delivery in food is their survival to the harsh conditions during the passage through the GIT. The encapsulation of cells could be one possibility to increase the cell’s stability in GIT conditions. Therefore we compared the stability of free and encapsulated cells in the conditions simulating those in GIT. In order to study the stability of capsules produced without emulsifier, 0.5% w/w of lecithin and 0.5% w/w of emulgator D, GIT conditions were simulated and samples collected for bacteria counting during 6 hours. The recommended number of viable cells in food, in the final product, is $10^6$ to $10^7$ CFU/g by the time the colon is reached, in order to be accepted as having a therapeutic role in the host, and daily consumption of 100 g of the product should also take place regularly (Madureira et al., 2010). This means that before ingestion the number of viable cells provided should be around $10^8$ to $10^9$ CFU/g. The initial cell concentration in pellets of bulk moist capsules at the beginning of the experiment was 8.5 log CFU mL$^{-1}$ for free cells, 9.0 log CFU mL$^{-1}$ for capsules with 0.5% w/w of lecithin and 8.0 log CFU mL$^{-1}$ for capsules with 0.5% w/w of emulgator D. This experiment was repeated three times for free cells and capsules with lecithin and twice for capsules with emulgator D, the duplications were made with independent capsules production and the mean and standard deviation were calculated and represented in Figure 3.9.

![Figure 3.9](image-url) Figure 3.9 – Simulation of GIT conditions and changes in viable cells count for free cells (blue), capsules with 0.5% w/w of lecithin (red) and 0.5% w/w of emulgator D (green) during simulated conditions of digestion. The samples were collected at time 0, 2, 4 and 6 hours and inoculated in MRS agar for 48 hours at 37°C. The first 2 hours simulate stomach conditions with pH 2 and pepsin. At 2 hours the pH is increased to 7 and bile salts and pancreatin were added to simulate intestinal conditions. The error bars represent the standard deviation.
The results were similar for capsules with lecithin and emulgator D, the final cell concentration was 4.6 log CFU mL\(^{-1}\) (50.8% of initial concentration) and 4.9 log CFU mL\(^{-1}\) (61.3% of initial concentration), respectively. After 2 hours incubation the decline in cell concentration for free cells is significantly higher (-60%) than for encapsulated cells (-0.6% for lecithin and -2.5% for emulgator D) and after 4 hours the cell concentration was 0.6 log CFU mL\(^{-1}\) (6.7% of initial concentration). Regarding these results, it is possible to conclude that both capsules with lecithin and emulgator D have a protective effect in *L. casei* cells. Since the capsules with emulgator D tend to form aggregates it was difficult to uniformly disperse them in the simulated solution and this can cause some variations in obtained results. However, considering the standard deviation, the result more accurate was obtained for emulgator D since the other formulations showed a higher standard deviation for the last 2 data points. Madureira, et al. (2010) studied the protective effect of whey cheese matrix (WCM) on *Lactobacillus casei* LAFTI L26, *Lactobacillus acidophilus* LAFTI L10 and *Bifidobacterium animalis* Bo and used MRS media as control. Also Pimentel-González and his co-workers (2009) studied the survival of encapsulated *Lactobacillus rhamnosus* LC705 in double emulsions formulated with sweet whey cheese, in GIT conditions. The results from these two studies are summarized in Table 3.2. After comparing the results obtained with capsules produced with lecithin and emulgator D, it is possible to conclude that although both capsules had a protective effect on *L. casei* cells, this effect was not so efficient as in the studies mentioned previously.
Table 3.2 – Results summary from the studies performed by Madureira et al., (2010) and Pimentel-González and co-workers (2009) about the protective effect of whey cheese matrix (WCM) on Lactobacillus casei LAFTI L26, Lactobacillus acidophilus LAFTI L10 and Bifidobacterium animalis Bo and the survival of encapsulated Lactobacillus rhamnosus LC705 in double emulsions formulated with sweet whey cheese, in gastrointestinal condition.

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Initial concentration (log CFU mL(^{-1}))</th>
<th>Intermediate concentration (log CFU mL(^{-1}))</th>
<th>Final concentration (log CFU mL(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free L. casei in MRS</td>
<td>9</td>
<td>≈ 0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Encapsulated L. casei in WCM</td>
<td>9.5</td>
<td>7</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Free L. acidophilus in MRS</td>
<td>9</td>
<td>-</td>
<td>4.4</td>
<td>(Madureira et al., 2010)</td>
</tr>
<tr>
<td>Encapsulated L. acidophilus in WCM</td>
<td>8.5</td>
<td>-</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Free B. animalis in MRS</td>
<td>7.8</td>
<td>-</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Encapsulated B. animalis in WCM</td>
<td>8</td>
<td>-</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Free L. rhamnosus</td>
<td>6.57</td>
<td>-</td>
<td>5.88</td>
<td>(Pimentel-González et al., 2009)</td>
</tr>
<tr>
<td>Encapsulated L. rhamnosus</td>
<td>6.74</td>
<td>-</td>
<td>8.66</td>
<td></td>
</tr>
</tbody>
</table>

3.4. Storage experiment

To evaluate the influence of encapsulation on cell’s viability and stability during long-term storage, capsules were incorporated into different dairy products and tested for several weeks. The concentration of viable cells in the product at consumption is an important parameter and the viable cells concentration may change during storage. Besides this cell concentration, other important parameters were measured according with the selected product.
Encapsulated *L. casei* stored in reconstituted reconstituted skim milk

Encapsulated *L. casei* were stored in reconstituted reconstituted skim milk at 4°C for 6 weeks. Every week the pH and viable cell concentration was measured in samples; the results are presented in Table 3.3.

Table 3.3 - Survival of *L. casei* as free cells or in capsules with 0.5% w/w of lecithin in reconstituted skim milk for 6 weeks at 4°C. The samples were collected once a week and inoculated in MRS agar for 48 hours at 37°C. The pH of each sample was measured during the 6 weeks for free and encapsulated cells. Two independent experiments were performed at the same time for each sample, same capsules production, and the mean were calculated; σ represents the standard deviation. For week 6, only one measure was performed for the sample with capsules.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Free cells</th>
<th>Capsules with 0.5% w/w of Lecithin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[L. casei] (log CFU mL⁻¹)</td>
<td>σ</td>
</tr>
<tr>
<td>0</td>
<td>8.4</td>
<td>0.07</td>
</tr>
<tr>
<td>1</td>
<td>8.4</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>8.6</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>8.6</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
<td>0.04</td>
</tr>
<tr>
<td>5</td>
<td>8.6</td>
<td>0.06</td>
</tr>
<tr>
<td>6</td>
<td>8.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Both free and encapsulated cells of *L. casei* survival well in reconstituted skim milk during 6 weeks, since the cell concentration was very stable for both. The number of viable cells slightly increased for both conditions which means that pH should decrease because *Lactobacillus* produces lactic acid. However, as see in Table 3.3, pH value increases during the experiment for encapsulated cells. In a study about viability of *Lactobacillus acidophilus* LaA3 and *Bifidobacterium bifidum* BB1 in several commercial fermented milks (Gueimonde et al., 2004), the milk was stored at 4°C for 30 days and cell concentration was above 10⁶ CFU mL⁻¹ for all of the 10 different products tested. The initial cell concentration of free *L. casei* was around 7 log CFU mL⁻¹ for all samples and after 30 days the number of viable cells was around 6.5 log CFU mL⁻¹ for the best result and 5.3 log CFU mL⁻¹ for the worst result. Nevertheless, is important to add that this case study cannot be totally compared to the present study since the product where cells were stored is not the same; for example, the pH is probably lower in fermented milk than in reconstituted skim milk solution, affecting cells survival and furthermore, aw of dehydrated milk is certainly much lower.
Encapsulated *L. casei* stored in yogurt

Yogurt was produced to test the stability of encapsulated *L. casei* during storage for 6 weeks at 4°C. During the experiment, once a week, the viable cell number was counted and the pH measured in samples as it is possible to see in Table 3.4.

Table 3.4 - Survival of *L. casei* as free cells or in capsules with 0.5% w/w of lecithin in yogurt for 6 weeks at 4°C. The samples were collected once a week and inoculated in MRS agar with vancomycin for 48 hours at 37°C. The pH of each sample was measured during the 6 weeks for free cells and capsules. Two independent experiments were performed at the same time for each sample, same capsules production, and the mean was calculated; σ represents the standard deviation. For week 5 only one measurement was performed for the sample with capsules and for week 6 only one measurement was performed for both free and encapsulated cells.

| Time (weeks) | Free cells | | Capsules | |
| --- | --- | --- | --- | --- | --- | --- |
|  | [L. casei] | σ | Yogurt pH | σ | [L. casei] | σ | Yogurt pH | σ |
| 0 | 8.66 | 0.05 | 4.45 | 0.07 | 8.81 | 0.03 | 4.52 | 0.09 |
| 1 | 8.65 | 0.05 | 4.45 | 0.07 | 8.65 | 0.09 | 4.52 | 0.09 |
| 2 | 8.69 | 0.03 | 4.17 | 0.01 | 8.65 | 0.03 | 4.15 | 0.02 |
| 3 | 8.65 | 0.06 | 4.09 | 0.01 | 8.65 | 0.02 | 4.08 | 0.04 |
| 4 | 8.75 | 0.04 | 4.06 | 0.01 | 8.71 | 0.17 | 4.05 | 0.04 |
| 5 | 8.50 | 0.02 | 4.13 | 0.04 | 8.60 | - | 4.09 | - |
| 6 | 8.79 | - | 4.10 | - | 8.65 | - | 4.13 | - |

The results presented in Table 3.4 show that, like in milk, both free cells and capsules are very stable when stored in yogurt. The pH decreases during the 6 weeks as it would be expected because of post-acidification. Usually, the yogurt starter cultures are active even when refrigerated and produce small amounts of lactic acid by fermentation of lactose which results in decrease of pH. Kailasapathy (2006) studied the survival of free and encapsulated *Lactobacillus acidophilus* DD901 and *Bifidobacterium lactis* DD920 during 6 weeks and also measured pH value. The initial pH was 4.52 for free cells and 4.48 for encapsulated cells and after 6 weeks the pH was 4.21 and 4.25 for free and encapsulated cells, respectively. Like the results in Table 3.4, the results from Kailasapathy are according with expectations of decreasing pH caused by post-acidification. In Kailasapathy studies, for cell survival in yogurt, at the beginning of the experiment the viable number for free cells was around 7.5 log CFU mL⁻¹ and 7.4 log CFU mL⁻¹ for encapsulated cells and, after 6 weeks, these concentration decreased to 3.3 log CFU mL⁻¹ for free cells and 5.2 log CFU mL⁻¹ for encapsulated *L. acidophilus* cells. In our case, the encapsulation does not affect the cells survival in a significant way because free cells are already very stable, although, in the study made by Kailasapathy it is possible to conclude that capsules have a protective effect on probiotic cells. Another possible explanation for the better stability of *L. casei* than *L. acidophilus* is the use of casein in the encapsulation process and its presence in dairy products; also the capsules produced by Kailasapathy were encapsulated with...
calcium-induced alginate polymer containing starch which can also affect the stability of the probiotic strain since the structure is different.

**Encapsulated *L. casei* stored in cheese**

Cheese has a pH value higher than yogurt and also higher total solids content which can offer better protection for probiotic cells like was mentioned in section 1.3.2. The cheese was stored at 10°C for 4 weeks and once a week the cell number was counted in samples and pH and water activity measured. The results are presented in Table 3.5; 3rd week measurements were not made because free and encapsulated cells slightly decreased in the previous weeks. According with the results in Table 3.5, we can conclude that the viable cell concentration was stable both in free and encapsulated cells since the initial and final cell numbers in the samples were almost the same. The final value for pH was almost the same as in the beginning of the experiment and for encapsulated cells the final pH was higher than in the beginning. It would be expected that the pH value would be stable because the cell number decreased which should decrease the production of lactic acid. Mirzaei et al. (2012) studied the survival of *Lactobacillus acidophilus* La5 in Iranian white brined cheese during 182 days. The initial cell number was 10 log CFU mL⁻¹ for free cells and 12 log CFU mL⁻¹ for cells encapsulated with calcium alginate and resistant starch. After 28 days of storage, the cell concentration for free cells was 12 log CFU mL⁻¹ and 5.1 log CFU mL⁻¹ after 182 days. For encapsulated cells their concentration was 12 log CFU mL⁻¹ after 28 days and 11 log CFU mL⁻¹ after 182 days. It is possible to assume that capsules did not play a significant role in cell protection during storage since free cells were already stable. It would be interesting to prolong our own storage experiment for more weeks to verify if the cell number would evolve as in the study with Iranian white brined cheese.

As we can observe in Table 3.5, the dry matter content has decreased during storage because cheeses were stored in brine solution. The absorption of this solution made the cheeses softer than in the beginning and alters the concentration values for *L. casei* cells calculated in Table 3.5. Therefore, the real concentration of *L. casei* cells were recalculated considering the dry matter content, for free and encapsulated cells, and are presented in Table 3.5 in blue. The new concentration values are not very different for time 0 but significantly different for the last data point. However, the general results remain the same, both free and encapsulated cells are stable and their concentration slightly decreases during storage. The *a_w* values also decreased during storage due to the salt diffusion from the brine into the cheese. Also caused by salt diffusion, the *a_w* is slightly lower inside the cheese than in the surface and the *a_w* total remained slightly above the general lower limit for bacterial growth (*a_w=0.9*).
Table 3.5 - Survival of free cells and encapsulated cells with 0.5% w/w of lecithin in cheese for 4 weeks, at 10°C. The samples were collected once a week and inoculated in MRS agar for 48 hours at 37°C. For each sample the pH, viable cell number and water activity were measured for the 4 weeks. For the first and last sample the dry matter content was measured by two different methods and the mean was calculated. The numbers in blue represent the real cell concentration, considering the dry matter content.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Free cells</th>
<th></th>
<th>Capsules</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[L. casei] (log CFU g⁻¹)</td>
<td>pH</td>
<td>Water Activity</td>
<td>Dry matter content (%)</td>
<td>[L. casei] (log CFU g⁻¹)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Surface</td>
<td>Inside</td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>0</td>
<td>9.1/9.4</td>
<td>5.16</td>
<td>0.96</td>
<td>0.96</td>
<td>0.98</td>
</tr>
<tr>
<td>1</td>
<td>9.0</td>
<td>5.35</td>
<td>0.93</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>8.9</td>
<td>5.31</td>
<td>0.93</td>
<td>0.93</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>8.7/9.3</td>
<td>5.17</td>
<td>0.93</td>
<td>0.93</td>
<td>0.92</td>
</tr>
</tbody>
</table>
3.5. Metabolic activity

Lactic acid bacteria are known as producers of lactic acid as a major or unique product of fermentative metabolism. Thereby lactic acid concentration and pH during incubation can be indicators of metabolic activity. Free and encapsulated cells of *L. casei* were incubated in MRS broth at 37°C for 48 hours and samples collected for bacteria counting and for measuring pH and lactic acid concentration in the medium at 0, 24 and 48 hours. The goal of this experiment was to assess if the encapsulation process had any influence in *L. casei* metabolic activity, the results are shown in Table 3.6.

**Table 3.6** – Study of metabolic activity for free cells and encapsulated of *L. casei* with 0.5% of lecithin. Cells were incubated for 48 hours at 37°C, samples were collected at 0, 24 and 48 hours to measure the number of viable cells, pH and lactic acid concentration.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Free cells</th>
<th>Capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(log CFU mL⁻¹)</td>
<td>(g/L)</td>
</tr>
<tr>
<td>0</td>
<td>7.5</td>
<td>5.56</td>
</tr>
<tr>
<td>24</td>
<td>9.4</td>
<td>3.85</td>
</tr>
<tr>
<td>48</td>
<td>9.3</td>
<td>3.64</td>
</tr>
</tbody>
</table>

After 24 hours of incubation both free cells and encapsulated cells have multiplied, decreasing the media pH due to increasing lactic acid concentration. At this point the values of pH and lactic acid are close for both inocula even though there were higher concentrations for free cells at time 0 hours. After 48 hours, the viable cell concentration and pH decreased for free cells even though the lactic acid has increased. For encapsulated cells the cell concentration was stable like pH but lactic acid concentration also increased. Overall, the measured parameters have similar values for free and encapsulated cells which means that the encapsulation process does not affect the cells metabolism.
3.6. Scale-up

In order to study the possibility of scale-up, the encapsulation process was performed in special equipment that can be seen in Figure 3.10. The main equipment was a stirred vessel where it was possible to perform the emulsification step with a batch capacity of up to 3.5 L. The experiments performed in this device were preliminaries studies and it should be tested more times.

Figure 3.10 – Equipment to perform the emulsions for the encapsulation process with increased oil volume. Scheme representing the set up: 1-entrace of the vessel; 2-stirrer; 3-vessel with 4.75 L of volume; 4- Erlenmeyer to collect the capsules and the oil. The arrows indicate flow circulation.

The encapsulation performed in the high capacity vessel was made without *L. casei* cells because the goal was to assure that it was possible to form capsules. For encapsulation 2 L of oil, without emulsifier, were emulsified with 200 g of reconstituted skim milk solution 35% (w/w). After collecting the emulsion with oil and milk, a microscopy preparation was made to inspect the formation of the capsules.
The capsules formed in this process (see Figure 3.11) were smaller than capsules produced in smaller scale with oil and without emulsifier since capsules appear to be around 20 or 30 µm of diameter and with spherical shape. To determine the viability of scaling-up the encapsulation process it is important to make more experiments. The main drawback of the equipment used was the undesired heating of the oil. In the encapsulation process, the milk has to be emulsified in the oil for 5 minutes at 5°C which means that the vessel, which is at room temperature, needs to be previously cooled to achieve such a low temperature before the emulsification starts. The attempt to keep the cold temperature in the vessel was not successful and unfortunately the temperature during the emulsification was maintained around 12°C. Also, the maximum agitation speed achieved was 400 rpm (Re=104 at 5°C, Re=469 at 40°C) instead of the usual 500 rpm. Therefore the cooling process needs to be optimized since the low temperature in the emulsion is an important step to the formation of capsules. Besides, in order to scale-up the process, it would be necessary to provide aseptic conditions.

In conclusion, it is possible to say that the encapsulation process could be scaled-up but more studies are required to have conclusive data.
4. Conclusion and future perspectives

After the optimization of the encapsulation process it was possible to conclude that the agitation speed was not relevant to the size and shape of the capsules produced with emulsifier, with the range of speeds tested. For capsules without emulsifier the effect of the agitation speed was more significant for the volume based median diameter of the capsules since for higher agitation speed the diameter was lower (-34%). The best size achieved for the diameter of capsules was around 52 µm for 0.5% w/w of lecithin and the capsules were spherical and white. For formulations with 4% w/w of lecithin and 0.5% w/w of emulgator D, the capsules were smaller however they were brown, in the first case, or formed aggregates in the second.

Due to the experiment to measure the metabolic activity it was clear that the encapsulation process did not affect the metabolic activity of the L. casei since the results were similar for free and encapsulated cells.

In what concerns the protective effect of capsules, they were very important in GIT conditions. In this experiment there was a higher cell survival for encapsulated cells with 0.5% w/w of lecithin and 0.5% w/w of emulgator D than for free cells, although the recommended values were not achieved. A possibility to overcome this problem was to increase the probiotic cells concentration in the beginning of the encapsulation process. However, in the storage experiment it was possible to conclude that capsules did not play an important role in protection. Both free cells and encapsulated cells were very stable in milk, yogurt and cheese during the time the experiment was performed. The usual expiration date for yogurt is 4 to 6 weeks, for fresh pasteurized milk is 10 days, for milk with extended shelf-life usually 4 weeks and for white brined cheese, when properly stored in brine or in milk and refrigerated, can last up to three months. Therefore, we can conclude that the storage experiment for the cheeses should continue for a longer period.

The process scale-up seems to be viable but more studies are required.

For future studies, a lot of issues should be considered. In the first place it is necessary to optimize the encapsulation formula in order to achieve higher survival rates results in GIT conditions. The results obtained for L. casei encapsulated by means of rennet-gelation in GIT simulation conditions were good but did not achieve the values recommended by WHO since it should be at least $10^6$ to $10^7$ CFU/g by the time the colon is reached and in this experiment the results were around $10^5$ CFU/g.

In second, it would be interesting to repeat these experiments with a different probiotic strain and the application of capsules with L. casei in non-dairy products. It would be interesting to find if the high survival rate obtained for L. casei in storage experiments was related with the use of dairy products, containing casein and other milk products, or to the strain in use. These experiments will show how the L. casei survives in non-dairy products and how other probiotic strains behave in the same conditions used for L. casei.

Finally the scale-up experiments performed were in a preliminary stage and should be developed to conclude if the process could be viable at industrial scale. It is necessary to overcome the problem
caused by the increase in fluid temperature in the vessel; it is necessary to find an efficient way to cool down the fluid for 5 minutes and then heat it until 40°C within the vessel. It is also necessary to provide aseptic conditions that could be applied in the process scale-up.
5. References


emulsifier and survival in simulated gastrointestinal conditions. *Food research International*, 42, 292-297.


6. Appendices

6.1. Reynolds number of stirrer

Oil density

Table 6.1 – Measurement of oil density at different temperatures by weighting an exact amount of oil at a certain temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Density (g/cm³)</th>
<th>Density (kg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.923</td>
<td>923.0</td>
</tr>
<tr>
<td>10</td>
<td>0.924</td>
<td>924.4</td>
</tr>
<tr>
<td>20</td>
<td>0.910</td>
<td>909.6</td>
</tr>
<tr>
<td>35</td>
<td>0.899</td>
<td>899.2</td>
</tr>
<tr>
<td>40</td>
<td>0.895</td>
<td>894.7</td>
</tr>
</tbody>
</table>

Viscosity

Table 6.2 – Shear viscosity of oil at different temperatures determined on rotary viscometer Kinexus in the coaxial cylinders geometry C25/PC25 DIN.

<table>
<thead>
<tr>
<th>Temperature(°C)</th>
<th>Shear viscosity(Pa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.148</td>
</tr>
<tr>
<td>10</td>
<td>0.113</td>
</tr>
<tr>
<td>15</td>
<td>0.088</td>
</tr>
<tr>
<td>20</td>
<td>0.070</td>
</tr>
<tr>
<td>25</td>
<td>0.056</td>
</tr>
<tr>
<td>30</td>
<td>0.046</td>
</tr>
<tr>
<td>35</td>
<td>0.038</td>
</tr>
<tr>
<td>40</td>
<td>0.032</td>
</tr>
<tr>
<td>45</td>
<td>0.027</td>
</tr>
</tbody>
</table>
Figure 6.1 – Shear viscosity of the oil measured at different temperatures.

**Reynolds number of stirrer**

Stirrer diameter = 5 cm  
Agitation speed = 500 rpm

**Table 6.3** – Calculation of the Reynolds number of stirrer for the oil at 5°C and 40°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cinematic viscosity (m²/s)</th>
<th>Reynolds Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.6E-04</td>
<td>130</td>
</tr>
<tr>
<td>40</td>
<td>3.6E-05</td>
<td>587</td>
</tr>
</tbody>
</table>